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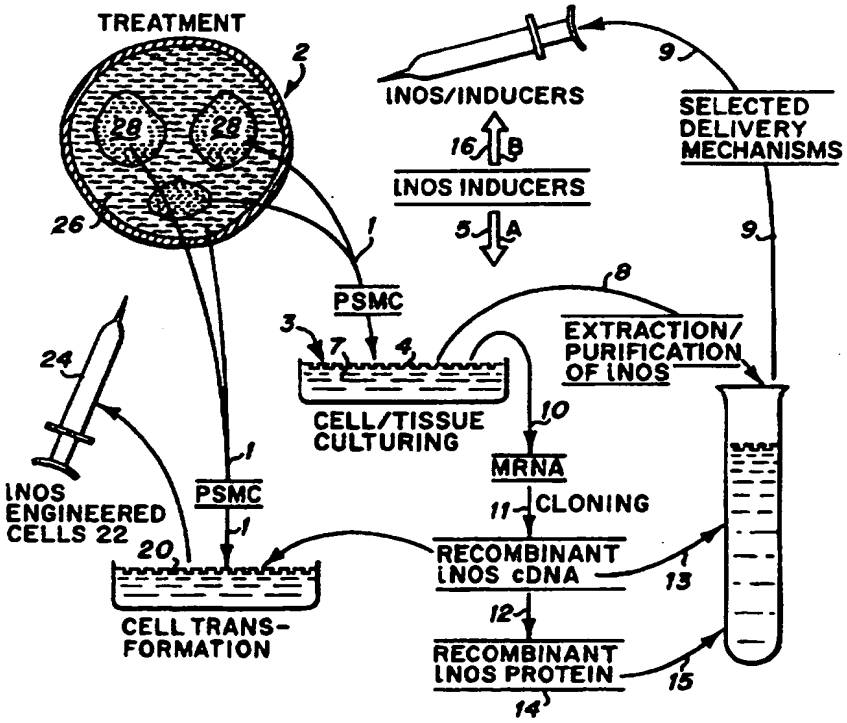
(54) Title: AMELIORATION OF HUMAN ERECTILE DYSFUNCTION BY TREATMENT WITH iNOS, AND RELATED NOS AGENTS

(57) Abstract

Treatment of erectile dysfunction comprising administering to a patient, inducible Nitric Oxide Synthase (iNOS) agents, including penile iNOS, inducers of penile iNOS, iNOS cDNA, or penile smooth muscle cells or corpora cavernosa with iNOS cDNA. Typical *in vivo* treatment involves delivery of these agents to the penile tissue of a patient by constant or intermittent implanted or external infusion pump, pellets, intrauretheral administration, injection or other related procedures. The genetically engineered cells or penile tissue from the patient hyperexpressing iNOS is implanted in microcapsules, pellets, or other methods, or directly by surgical inoculation into the corpora cavernosa. In certain cases, an oral or injectable systemic route of administration is applicable. Also disclosed are methods of treatment involving *in vitro* induction of iNOS in cultured smooth muscle cells and thereofter delivery of purified or recombinant iNOS enzyme, production of iNOS cDNA and genetic transformation with iNOS cDNA, followed by delivery thereof to the penis of a patient. The methods of this invention include hyperexpression and/or biological modulation of other endogenous and exogenous NOS isoforms in the penis, for the treatment of erectile dysfunction.



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TITLE: AMELIORATION OF HUMAN ERECTILE DYSFUNCTION BY TREATMENT WITH iNOS, AND RELATED NOS AGENTS

DESCRIPTION

TECHNICAL FIELD:

This invention relates to a treatment of erectile dysfunction, and more specifically to the treatment of erectile dysfunction by use of inducible Nitric Oxide Synthase (iNOS) agents, including iNOS, NOS isoforms, inducers of iNOS, iNOS cDNA and recombinant iNOS cDNA-transformed penile smooth muscle cells (iNOS engineered cells), for the purpose of ameliorating erectile vasculogenic dysfunction in the penis, typically vasculogenic dysfunction. The iNOS is induced in vivo e.g., in penile corpora cavernosa, to produce penile tissue specific expression of increased levels of iNOS for amelioration of the patient's condition. Or iNOS may be induced in vitro in excised and cultured corpora cavernosa cells, extracted, purified and thereafter provided to patients in a wide variety of delivery systems. Alternatively, recombinant iNOS can be synthesized in vitro and delivered to the patient for treatment. cDNA iNOS may be introduced in patient penile tissue to produce recombinant iNOS in vivo. In another alternative the ex vivo cultured cells can be transformed with penile iNOS and/or NOS isoform cDNA, and the resultant genetically engineered cells introduced into the corpora cavernosa in vivo. Other NOS isoforms or their respective cDNAs and recombinant proteins, and penile cells transfected by the NOS cDNAs may be employed using the same procedures described for iNOS.

BACKGROUND ART:

Impotence, the inability to obtain or maintain a penile erection sufficient for sexual intercourse, afflicts more than 12 million men in the USA. It is associated with aging and occurs in 25% of men aged 65, and 55% of men aged 75, irrespective of the fact that the libido of the majority of these patients is relatively unaffected. Annually it results in more than 400,000 outpatient visits and 30,000 hospital admissions in the U.S.. Surgical implantation of penile prosthesis increased from 19,000 in 1980 to 32,000 in 1989. Accordingly, the costs of treating impotence in 1989 is conservatively estimated at 250 million dollars. In human terms, although impotence is not usually a life-threatening situation, its consequences for the patient and his partner are psychologically very serious.

Contrary to earlier assumptions in the literature, as much as 90% of impotence is due to organic and not to psychogenic causes. Despite the fact that aging is a predisposing factor, organic impotence may be present as early as puberty in some patients. Vasculogenic and neurogenic alterations leading to penile erectile dysfunction are at the root of the majority of the organic impotence syndromes, since male hormone disturbances and other possible physical causes of loss of the libido play a minor rol in these problems.

Vascular disease of many causes will eventually lead to impaired penile erection. Thus,

atherosclerosis, certain types of hypertension, diabetes, heavy smoking and alcoholism are all recognized risk factors for erectile dysfunction. Diabetics as a group are the most prone to vasculogenic impotence with more than 50% incidence in a population of about 2.5 million in the USA.

This invention is based on the discovery that effective ameliorative treatment can be based on inducing the penile tissue-specific expression of Nitric Oxide Synthase (NOS), the enzyme which synthesizes the compound nitric oxide (NO), or expressing in vivo the recombinant NOS protein, which NO in turn functions as a main mediator of penile erection.

The physiology of normal erection can be divided into three distinct processes acting in concert: (a) increased arterial inflow; (b) decreased venous outflow; (c) active cavernosal smooth muscle relaxation. The latter appears to be the key event, but the penile blood vessel hemodynamics is also mediated by the smooth muscle of the arterial tree. Accordingly, active smooth muscle relaxation in the penile artery and sinusoids is considered to be the pivotal step in generating a normal erection. Abnormalities in penile smooth muscle function may be the critical site in erectile dysfunction.

In a normal erection the stimulation is transmitted to the penis through the nervi erigentes, the pelvic autonomic nerve fibers. Neurotransmitters are released from three systems: (a) norepinephrine from the sympathetic adrenergic fibers; (b) acetylcholine from the parasympathetic cholinergic fibers; and (c) a substance from the nonadrenergic-noncholinergic (NANC) fibers. The NANC neurotransmitter has been shown to be nitric oxide (NO) and to act upon the smooth muscle to cause relaxation.

The smooth muscle relaxation of the trabeculae surrounding the lacunar spaces of the corpora cavernosa has three important functions: (a) reduction of the normally high resting (flaccid) resistance to arterial flow, thus increasing this flow through the helicene arteries into the endothelium-lined lacunar spaces; (b) regulation of blood storage into the penis, allowing penile engorgement; and (c) transmission of approximately 80% of systolic blood pressure into the cavernosal space. The latter will compress the draining venules that run in parallel between the expanding smooth muscle and the tough inelastic tunica albuginea, resulting in venous outflow restriction. Detumescence occurs by a reversal of this process mainly by sympathetic control, that is, an increase of the tone of the smooth muscle in both compartments leading to reduction of arterial inflow and the size of the lacunar spaces, followed by venous runoff.

NO was identified as the vasoactive compound in the endothelium-derived relaxing factor (EDRF) and appears to play multiple roles in different biological processes. EDRF is released from the endothelial lining of blood vessels and induces different effects in hypoxia, vascular disease, septic shock, and inflammation. NO plays a significant physiological role in the maintenance of vascular tone by inducing locally the relaxation of the smooth muscle cells. Our work has shown that in the penile corpora cavernosa, NO is the main compound responsible for erection and appears to be the NANC neurotransmitter in the penis.

In the case of the penis, we have previously demonstrated by electric field stimulation (EFS), pharmacological treatments and the use of specific NOS inhibitors, that NO is the main mediator of penile erection in the human, dog, rat, and rabbit. A number of other laboratories have confirmed and extended these findings, by applying essentially two approaches: a) relaxation of corpora cavernosa strips in organ bath; b) erectile response in animal models. More recently, we have clearly shown the correlation between

erectile dysfunction in the rat model associated with impotence risk factors for men, and the reduction of penile NOS content.

One of the sites of NO release in the penis appears to be at the non-adrenergic non-cholinergic (NANC) nerve terminals of the corpora cavernosa, from which it diffuses and then binds to guanyl cyclase in adjacent cells and stimulates the formation of cGMP mainly in the smooth muscle target tissue. This cGMP synthesis in turn results in a decrease in intracellular $Ca2^{+}$, and subsequent smooth muscle relaxation and penile vasodilation.

It is known in the art that Nitric Oxide Synthase (NOS) is the enzyme catalyzing the formation of NO in endothelial cells, macrophages, brain, liver and several other cell types and tissues. There are two types of NOS: constitutive NOS (cNOS), whose levels do not appear to change upon different experimental conditions; and inducible NOS (iNOS), whose synthesis can be stimulated by bacterial toxins and certain growth factors. In general, cNOS is classified in several groups: a first is present in brain (one isoform of which is known as neuronal NOS, nNOS, or Type I NOS). A second cNOS is present in endothelial cells (endothelial NOS, eNOS or Type III NOS). iNOS (Type II NOS) was known to be expressed upon induction in macrophages, lung, liver, smooth muscle cells from large arteries and also in endothelial cells.

cNOS and iNOS isolated from different tissues show the existence of several isoforms within each group with specific cofactor requirements, mRNA sizes, and immunological properties. More than one isoform may be expressed simultaneously in the same cell type. (The different type designations are shown below in parenthesis.) Within the cNOS group, characterized by their Ca^{2+} dependence, there are three different cytosolic isozymes: A, present in the brain, cerebellum, and neuroblastoma cells; B, present in endothelial cells; and C, present in neutrophils. The first two are calmodulin dependent, and the third is calmodulin independent. Ib does not have BH^{4} and FAD as cofactors, and Ia is the only one using FMN additionally as cofactor. There is also a particulate Ca^{2+}/calmodulin dependent isoform that makes up over 90% of endothelial cell cNOS.

Within the iNOS group (principally Ca^{2+}/calmodulin independent with unknown regulators), a soluble type is produced upon induction by macrophages, hepatocytes, Kupfer cells, fibroblasts, endothelial cells, lung and livers in a variety of animal species. A different particulate type is present in the macrophages and is only NADPH dependent.

Further evidence for the significant difference between cNOS, nNOS and iNOS stems from the difference in their respective kinetics and substrate/cofactors requirements. L-arginine and NADPH are the common substrate and cofactor respectively. As noted above, the cNOS and iNOS isozymes can be distinguished in that cNOS is Ca^{2+}/calmodulin dependent, while iNOS is Ca^{2+} independent or only partially dependent. The reaction catalyzed by NOS is the conversion of L-arginine into L-citrulline, where NO is the product. NOS activity is inhibited by a series of competitive inhibitors such as N^{G}-nitro-L-arginine and N^{G}-methyl-L-arginine, or by NO chelators, such as hemoglobin. (3-H)citrulline synthesis is increased in certain cells by N-methyl-D-aspartate (NMDA) and glutamate. There are no really specific chemical inhibitors of the different NOS isoforms. Hydroxy-arginine and arginine dipeptides are also NOS substrates. Aminoguanidine appears to be a preferential inhibitor for iNOS, but its specificity varies with the cell type.

In addition, NOS mRNA (and resulting cDNA) are isoform specific and are expressed in a tissue differential fashion. For example, the DNA sequences (including introns and exons) of the gene for rat and human brain cNOS (nNOS) have been cloned. Their mRNA is expressed as a 10.5 kb polynucleotide species. But the same mRNA species is not found in rat kidney, liver, or heart tissue. Besides rat cerebellum, nNOS has been purified from rat polymorphonuclear neutrophiles and other sources. The iNOS cDNA has been cloned from bovine and human endothelium. In the latter case, the corresponding cNOS mRNA is 4 kb in length, and is encoded by a gene different from that expressed in the brain. Further, iNOS has been cloned from LPS-stimulated rat and mouse macrophages, rat vascular smooth muscle, human hepatocytes and condrocytes. Although only one iNOS gene has so far been identified in each of the species investigated (human, rat, mouse), recent evidence suggests that some of the repeated sequences identified in the human genome may correspond to two or more different genes.

Induction of non-penile NOS is triggered in vivo in rat and mouse models by injection of lipopolysaccharide from E. Coli (LPS), and in vitro by incubation of cells or tissue strips with LPS, interleukin β, tumor necrosis factor (TNF-α) and/or γ interferon. The induction is protein synthesis-dependent and blocked with dexamethasone or other glucocorticoids, and with TGF-β.

The presence of NOS in the human, rabbit, and rat penis tissue homogenate has been shown by us by following the conversion of (3-H) L-arginine into (3-H) L-citrulline in the cytosol fraction. We and others have detected the nNOS isoform by Western Blot, and localized it in the nerve terminals of the penis by histochemistry and immunocytochemistry. However, no characterization has yet been made of the main penile NOS isozyme responsible for NO synthesis during sexual stimulus. In addition, recent gene knock-out experiments failed to affect the reproductive behavior of transgenic mice when nNOS, eNOS or iNOS were silenced. This paradoxical result appears to derive from the expression or activation of non-NO alternative erectile pathways. Our own current work described herein indicates the existence of distinctive penile NOS isozymes different from those in other tissues. Other non-NOS dependent pathways may be present in the penis and they are supposed to cooperate during penile erection with the NO cascade, or become predominant after a long-term impairment or silencing of the penile NOS. These putative physiological ancillary relaxants of the penile smooth muscle include vasoactive intestinal polypeptide (VIP), calcitonin gene related polypeptide (CGRP), prostaglandins, etc.

That NOS decrease or inactivation may be associated with certain forms of erectile dysfunction has been shown by our recent work on aged intact rats, diabetic BB rats, and androgen-depleted rats. In both intact senescent rats and castrated rats, the levels of erectile response to EFS and of penile NOS can be restored to normal values by androgen administration. EFS itself modulates (activates or inhibits) penile NOS activity, and it does this in a differential form between intact and castrated rats.

However, no treatment based on the manipulation of endogenous NO synthesis, or endogenous NOS activity or expression, has been shown or proposed in the scientific literature. The current pharmacotherapy of erectile dysfunction is based exclusively on the topical application, or the direct intermittent self-injection, or intraurethral administration into the penile corpora cavernosa of mixes of vasoactive compounds, including nitrodonors, immediately prior to sexual intercourse, or surgical treatments based on prosthesis implantation or arterial/venous operations. For example, U.S. Patent Nos.

4,931,445 (**Goldstein et al.**), 5,336,678 (**Cavallini**), and 5,278,192 (**Fung et al.**), teach methods of treating impotence through administering the drugs etoparidone, Minoxidil, or isobutyl or isoamyl nitrite, respectively. **Bredt et al.** in U.S. Patent 5,268,465 have characterized a rat brain cDNA encoding a calmodulin-dependent NOS molecule of specific sequences, but does not suggest or teach treatment of erectile dysfunction therewith. This is a cNOS or nNOS. **Stuehr et al.** in U.S. Patent 5,132,407 teach a three-component calmodulin-independent NOS flavoprotein purified from mouse macrophages, but does not teach treatment of erectile dysfunction.

Voss et al. in U.S. Patent 4,801,587 teaches use of DMSO as an absorption agent to introduce papaverine, a compound known for treatment of human impotence. **El-Rashidy** in U.S. Patent 5,256,625 teaches the use of hydroxy propyl-β-cyclodextrin as an absorption enhancer for papaverine.

The main subject of the current invention, the penile iNOS isoform, has previously never been detected either at the enzymatic or protein levels, at the mRNA levels, nor in penile tissue sections by immunocytochemical procedures. Other than our own exclusive work on rat penile smooth muscle cells (RPSMC), human penile smooth muscle cells (HPSMC), on tissue slices of human and rat corpora cavernosa, and in vivo in the rat corpora cavernosa described herein, there are no reported studies on iNOS detection in penile cells. It was unknown whether iNOS has any physiological role in erectile function, and there are no publications proposing or suggesting that penile iNOS ever existed or could be applied for the therapy of erectile dysfunction.

Indeed, vascular iNOS, when induced, may have a deleterious effect on blood pressure. It is assumed to participate in septic shock, without apparently acting on the normal maintenance of blood vessel tone. In addition, the induction of iNOS, iNOS cDNA or gene therapy to improve penile erection has not been considered before probably because of the risk of systemic hypotension or uncontrollable priapism, and the belief that it does not participate in the natural erectile response. To our knowledge, no publications on the continuous delivery of compounds into the penis are available as opposed to intermittent treatment modulators. No publications teach the use of the other NOS isoforms (neuronal and endothelial) or their regulation for treating erectile dysfunction.

Accordingly, there is a need in this field to provide an improved method of treatment of erectile dysfunction by inducing endogenous production of iNOS in penile tissue or by introduction of exogenous iNOS to penile tissue, and in the alternative, the application of other NOS isoforms or their regulation in the penis by applying the procedures of this invention.

DISCLOSURE OF INVENTION:

It is among the objects and advantages of this invention to provide methods and compositions for amelioration of erectile dysfunction through localized treatment of the penis with penile iNOS and/or other NOS isoforms, NO protein(s), inducers of iNOS, and iNOS cDNA, in order to minimize the systemic effects.

Other objects and advantages include to:

Provide methods and compositions for increasing the level of iNOS in patient penile tissue through direct or indirect introduction of penile iNOS into the tissue;

Provide delivery systems for introduction of inducers of iNOS into th penile tissue of patients for the purpose of ameliorating vasculogenic impotence, including vasculogenic impotence;

Provide methods of cloning, sequencing and expression of penile iNOS, and evidence of its distinctive properties;

Provide methods of treating human patients exhibiting erectile dysfunction symptoms by a variety of systems, including: penile implantation of microcapsules containing iNOS inducers; or penile iNOS recombinant protein (native or modified) in the corpora cavernosa; or by genetically engineering in vivo production of iNOS in affected penile tissue, or ex vivo in penile cells and tissue to be implanted in the penis, or by systemic administration in certain cases; and

Provide compositions of penile and non-penile NOS isoforms and their biological regulators and methods of use in the treatment of erectile dysfunctions.

Other objects of this invention are evident from the Detailed Description, Drawings and Claims.

The present invention provides a new treatment for erectile dysfunction by the mechanism of raising the level of inducible nitric oxide synthase (iNOS) in the penis by various agents, which in turn, in vivo, effectuates the physiologically controlled production of nitric oxide (NO) to mediate the erectile response by its effect in relaxing the smooth muscle in the corpora cavernosa of the penis.

This invention is directed to methods and compositions for ameliorating erectile dysfunction in patients through increasing levels of iNOS in penile tissue either by direct introduction of iNOS to penile tissues, or, in the presently preferred embodiment, inducing endogenous production of iNOS by treatment with appropriate iNOS inducers, introduction of iNOS cDNA or by transformation of excised and cultured penile corpora cavernosa cells or tissue pieces with recombinant iNOS cDNA and re-introduction of these cells in the penis in vivo where they proliferate, and improve the NO-producing capability of the corpora cavernosa tissue. These procedures are also applicable to use of other NOS isoforms, and their corresponding cDNA, genetically engineered cells, or in vivo gene therapy approaches, in the penis to therapeutically modulate erectile dysfunction.

Figure 1 shows schematically the several embodiments of the invention. In one embodiment, cells or pieces from excised tissue specimen 1 (preferably corpora cavernosa cells) from penis 2 are cultured or incubated 3 in appropriate media. The cultured Penile Smooth Muscle Cells (PSMC) or corpora cavernosa slices 4 are then treated with inducers 5 appropriate for in vitro induction of iNOS. The iNOS 7 from the induced cells or tissue 4 can be extracted, purified 8 and delivered 9 to the penis 2 for treatment. Alternately, the mRNA from in vitro induced PSMC can be isolated 10 and its corresponding cDNA cloned 11, e.g., by RT/PCR (reverse transcription and polymerase chain reaction), library techniques, or other cloning techniques. The resulting iNOS cDNA 12 is prepared 13 and delivered 9 to the penis of a patient for treatment. Alternately, in the preferred embodiment, the cloned iNOS cDNA 12 can be used to generate recombinant iNOS protein 14 which can be recovered 15 and delivered 9 to the penis 2 for treatment. In yet another embodiment, the cultured PSMC cells or tissue pieces 20 are transformed with the recombinant penile iNOS cDNA 12 to produce iNOS engineered cells or corpora cavernosa tissue 22 which are then introduced 24 into appropriate penile tissue 26, such as the corpora cavernosa 28. There the cells or tissue implants proliferate to rejuvenate and augment the endogenous

iNOS producing capability of the penile cells and tissue structures. Inducers 16 appropriate for in vivo induction of iNOS can be delivered 9 to the penis 2 of the patient thus raising the level of iNOS endogenously produced in the penile tissue. The delivery of NOS biological agents to the penis can be achieved by systemic administration in certain cases, for example, as detailed in Example 10 below.

In vitro induction of iNOS in the cultured Rat Penile Smooth Muscle Cells (RPSMC), Human Penile Smooth Muscle Cells (HPSMC), and pieces of or rat or human corpora cavernosa was accomplished by treatment with different agents alone and in combination, such as lymphokines and bacterial lipopolysaccharide (LPS). Treatment with various inducer mixes caused significant increases in the NOS activity of the treated cells and tissue as measured by the accumulation of nitrites in the culture medium. The time-course for the RPSMC showed a linear response up to at least 60 hr, which was inhibited by L-NAME, thus indicating that this increase in nitrite release is due to iNOS induction. The fact that the observed stimulation of nitrite accumulation in the RPSMC culture medium was due to iNOS induction was confirmed by demonstrating the presence in the induced penile cells of both iNOS mRNA and iNOS protein by northern and western blotting respectively. The probe used for northern blot analysis was a 350bp fragment of iNOS cDNA from RPSMC; western blot analysis was by reaction of lysed induced RPSMC with a commercially available iNOS antibody.

Based on our results on the conditions for in vitro induction of cultured cells, treatment in vivo is accomplished by delivering an inducer mix, such as the one described in detail in Example 2 below comprising E. coli lipopolysaccharide (LPS), recombinant rat interferon-γ (IFN-γ), recombinant human tumor necrosis factor (TNF-α), and recombinant human interleukin-1 β (IL-1 β), to the penile corpora cavernosal tissue of a rat model. Application to human patients requires human IFN-γ. Other mixes may be used and added to the delivery of single agents. A delivery method involving constant infusion of the inducer mix by means of an osmotic pump attached to a catheter which feeds into the corpora cavernosal tissue is the presently preferred method, although any suitable delivery system can be employed, such as embedded or injected microcapsules, injection of the mix, intraurethral introduction, topical or subdural application, or the like.

The disclosed pump system can be set to deliver the inducer mix to a rat for a short period (e.g., 1 ul/hr during 3 days) or for a longer treatment period (e.g., 0.5 ul/hr during 14 days). We have observed that the short-term (ie., 3 day) treatment is more efficacious than the long-term (ie., 14 day) treatment, however this may be an artifact, due to the inactivation of some of the biological constituents in the inducer mix when the mix remains in the pump for extended periods. The formulation, delivery system, and scheduled administration should be adjusted for the human patient on a case by case basis.

The described treatment with iNOS inducers markedly improves the erectile response in in vivo tests performed on rats of three different age groups, adult (5 month old), "old" (20 month old), and "very old" (28-32 month old). The erectile responses of the subject rats after completion of the inducer treatment were measured by detecting the maximal intracavernosal pressure in response to electrical field stimulation (EFS) of the cavernosal nerve in the animals. No priapism was observed, indicating that the induced penile iNOS remains under physiological control. No hypertension or other major side effects were detected.

Treatment with a sub-optimal dose of the nitric oxide synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME) subsequent to treatment with the inducer significantly reduced the observed erectile response. This data demonstrates that the inducer treatment mechanism is via the NOS cascade. That the inducer treatment works by induction of nitric oxide synthase is further demonstrated by data that the penile tissue homogenates of rats treated with inducers of iNOS showed increased NOS activity relative to untreated controls, by histochemical detection of NOS activity and protein in penile tissue sections, and by Western Blot detection of iNOS in the penile cytosol.

The feasibility of iNOS induction in the rat corpora cavernosa is also demonstrated ex vivo in incubations of penile slices and determination of iNOS by nitrate and Western Blot assays. This process can also be effected by the presence of the invention ex vivo in incubations of human corpora cavernosa, as shown by similar assays, and in human penile smooth muscle cells (HPSMC) by RT/PCR of the extracted RNA.

A cDNA library was prepared from induced RPSMC, screened with adequate probes, and several $iNOS^+$ clones were detected and sequenced. Comparison of the complete sequence of the RPSMC iNOS coding region showed two distinctive amino acid differences as compared to the published sequence of rat vascular iNOS, suggesting a distinctive penile iNOS species. A construct of RPSMC iNOS was used to stably tranfect RPSMC and the cells constitutively expressed iNOS. A parallel sequence of the HPSMC iNOS was also obtained, confirming iNOS induction in human penile cells and the distinctive nature of the penile iNOS.

The rat PSMC iNOS construct was used for in vivo gene therapy of impaired corpora cavernosa in aged rats, showing that by a single injection of a liposome-based RPSMC iNOS construct preparation, several rats displayed after five days an erectile response higher than that typically observed even in adult rats. iNOS protein produced in vivo was detected by Western Blot. This improvement in erectile response was more uniform (all rats tested) and equally intense after 11 days after the single injection treatment. Another construct of RPSMC iNOS cDNA was prepared in a baculovirus expression vector and iNOS protein was expressed in insect cells. Both the transfected cells and tissue pieces, and the iNOS recombinant protein, can be used for increasing penile NO synthesis by direct molecular or implantation in the corpora cavernosa.

The methodology of this invention can be extended to other penile and non-penile NOS isoforms for these and their biological regulators for the treatment of erectile dysfunction.

BRIEF DESCRIPTION OF DRAWINGS:

The invention is described in more detail by reference to the drawings in which:

Fig. 1 is a schematic illustration of the methods of the invention including use of iNOS and iNOS inducers directly or indirectly to treat human erectile dysfunction;

Fig. 2 is a series of bar graphs showing the in vitro stimulation of nitric oxide synthesis in cultures of rat penis smooth muscle cells (RPSMC) by treatment with iNOS inducer mixes comprising one lymphokine supplemented with and without bacterial lipopolysaccharide (LPS) as measured by the accumulation of nitrites in the culture medium;

Fig. 3 is a series of bar graphs showing the in vitro stimulation of nitric oxide synthesis in cultures of RPSMC by treatment with iNOS inducer mixes comprising two or three lymphokines plus LPS as measured by the accumulation of nitrites in the culture medium;

Fig. 4 is a line graph showing the time course and stability of the iNOS induction of RPSMC cultures treated with a standard binary iNOS inducer mix in the presence or absence of the NOS inhibitor L-NAME as measured by accumulation of nitrites in the culture medium;

Fig. 5 is a bar graph showing that the turnover of the enzyme when the inducers are removed and not replaced is slow thus indicating the persistence of high levels of iNOS for a considerable period after induction;

Fig. 6 is a Northern Blot of poly A+ mRNA from RPSMC submitted to a time-course of induction, as hybridized with a RPSMC iNOS probe;

Fig. 7 is a Western Blot of an extract obtained by lysis of induced RPSMC and reacted with commercial iNOS antibody;

Fig. 8 is a line graph showing the uniform improvement in erectile response to electrical field stimulation of the cavernosal nerve in 5, 20, and 30 month old rats treated for three days with a constant infusion of several inducers of the inducible form of penile nitric oxide synthase of this invention;

Fig. 9 is a bar graph showing increased sensitivity of the erectile response of rats as treated in **Fig. 8** to a suboptimal dose of an inhibitor of nitric oxide synthase;

Fig. 10 is a line graph showing that a medium-term local treatment (14 days) of three age groups with inducers of nitric oxide synthase does not give a better response than the short term paradigm in th enhancement of penile erection triggered by electrical field stimulation;

Fig. 11 is a bar graph showing that a suboptimal dose of a nitric oxide synthase inhibitor differentiates respondents from non-respondents in the EFS erectile test in rats of different ages;

Fig. 12 is a bar graph showing the increase in nitric oxide synthase activity in the penile cytosol of rats treated for three days with inducers of nitric oxide synthase;

Fig. 13 is a bar graph showing that the increase in NOS activity in the cytosol of inducer treated rats is inhibited by treatment with L-NAME but is unaffected by treatment with aminoguanidine;

Figs. 14 A, B and C are photomicrographs of penile tissue showing the increase in NOS activity in the penile tissue of inducer-treated rats as detected by histochemistry;

Figs. 15 A, B and C are photomicrographs of penile tissue showing the increase in iNOS in the penile tissue of inducer-treated rats as detected by immunocytochemistry;

Fig. 16 is a Western Blot autoradiogram showing the expression of iNOS protein detected in the corpora cavernosa from aged rats subjected to pump infusion of iNOS inducers;

Fig. 17 is a Western Blot autoradiogram showing that iNOS induction can be obtained ex vivo in the penis, in incubations of slices of rat and human corpora cavernosa where iNOS increase is demonstrated by nitrite release and Western Blot assays;

Fig. 18 is a sequence overlap map showing the iNOS cDNA clones that were sequenced from a first induced RPSMC library;

Fig. 19 is a sequence comparison showing the iNOS cDNA clones that were sequenced from a second induced RPSMC library and the two clones selected for constructing the iNOS cDNA recombinant representing the whole coding region;

Fig. 20 is a comparative sequencing chart showing the strategy applied to sequence the HPSMC iNOS cDNA, and the comparison of 60% of the HPSMC iNOS coding region with that corresponding to human hepatocyte iNOS cDNA;

Fig. 21 is a chart showing the primers used for generating the RT/PCR fragments from HPSMC iNOS;

Fig. 22 is a chart showing the homology between the sequence of regions in the RPSMC iNOS cDNA and their counterparts in the human hepatocyte iNOS;

Fig. 23 are pressure plot charts showing the improvement of the erectile response to EFS in aged rats subjected to gene therapy by direct injection in the corpora cavernosa with a construct of RPSMC iNOS cDNA; and

Fig. 24 is a Western Blot analysis showing the expression of iNOS protein detected by Western Blots in penile cytosol from aged rats subjected to gene therapy by direct injection in the corpora cavernosa with an RPSMC iNOS cDNA construct.

BEST MODE(S) FOR CARRYING OUT THE INVENTION:

The following detailed description illustrates the invention by way of example, not by way of limitation of the principles of the invention. This description will clearly enable one skilled in the art to make and use the invention, and describes several embodiments, adaptations, variations, alternatives and uses of the invention, including what we presently believe is the best mode of carrying out the invention.

Measurement of NO production is based on the generation of NO co-products, such as (3-H) citrulline originated from (3-H) L-arginine, or from the relatively stable NO metabolites, such as nitrites and nitrates. The latter procedure is particularly suitable for measuring the release and accumulation of the NO-products in the extracellular medium.

iNos protein can be detected and evaluated by immunodetection in tissue sections (immunocytochemistry) and cytosol (Western Blot), by Northern Blot analysis of its mRNA, or by histochemical detection of NOS-associated activity (NADPH diaphorase assay).

EXAMPLE 1 **<u>In vitro Induction of iNOS in Smooth Muscle Cells</u>**

This example demonstrates the method for obtaining a high level of stable induction of nitric oxide synthase <u>in vitro</u> in cultures of rat penis smooth muscle cells (RPSMC), to select conditions for the <u>in vivo</u> induction experiments.

Primary cultures of RPSMC were initiated from small pieces of penile tissue excised from 3 months old Sprague Dawley rats, utilizing Dulbecco's modified Eagle medium (DMEM) with 20% fetal calf serum, at 37°C in the presence of 5% CO_2. Cells grown from the explants were then transferred to medium with 10% serum, and at the 4th to 10th passage (1:3 splits) they were utilized for the experiments, unless

otherwise indicated. Alternatively, cells from the 4-8th passage stored under liquid nitrogen were used to reinitiate the cultures, and used for incubations on the following 2-4 passages. These cultures are considered to consist mainly of smooth muscle cells from the penis based on morphological and cyto-immunochemical criteria.

All experiments were conducted at 90-100% cell confluence on "Primaria" cultureware, usually 24-well plates, adding or not the inducers indicated in each experiment. When not specifically stated, the standard binary induction mix consisted of bacterial lipopolysaccharide (LPS) (10 ug/ml) and recombinant murine interferon-γ (INF-γ) (250 U/ml). Other substances to be tested were added as indicated to the serum-containing medium, and the incubation proceeded for the times indicated. The medium was always collected and stored for an indirect measurement of NO production based on the determination of its conversion into nitrites. The procedure was based on the application of the Greiss reagent, using 250 ul of the medium mixed with an equal volume of reagent. Each experimental condition was carried out in triplicate.

Figure 2 presents the results obtained in 48 hr incubations with one cytokine supplemented or not with LPS. In the absence of inducers (panel 1, left bar) there is only a very marginal NO production (less than 5 uM, or 2.5 nmoles per well), and LPS by itself (1 and 10 ug/ml) only marginally stimulate this basal synthesis by less than 50% (panel 1, central and right bars, respectively).

The addition of INF-γ (50 to 500 U/ml) in the absence of LPS (panels 2 through 5, left bar) causes a dose-proportional moderate increase of nitrites to a maximum of 45 uM. Supplementation with 1 or 10 ug/ml LPS (central and right bars, respectively) stimulates the induction up to 120 uM, or nearly 20-fold the basal level. In contrast, TNF-α up to 500 u/ml did not have any effect either in the presence or the absence of LPS (panels 6 and 7). Interleukin-1β (IL-1β) at the minimum concentration tested (5 ng/ml) in the presence of 10 ug/ml LPS (panel 8, right bar) was slightly more effective than the 500 U/ml dose of INF-γ (panel 8, right bar), but higher concentrations of IL-1β up to 100 ng/ml (panels 9, 10), enhanced relatively little the level of stimulation.

The supplementation of the INF-γ/LPS binary combination with additional cytokines (tertiary or quaternary mixes) is presented on **Figure 3**. The basal NO synthesis in this experiment in the absence of cytokines (panel 11) was slightly higher than in the previous series (compare with panel 1), and the same occurs with the binary combination of 10 ug/ml LPS and 250 U/ml INF-γ (panel 12 compared with panel 5), chosen as "standard mix" for successive in vitro experiments because of its low cost. Taking this mix as reference, the addition of TNF-α to 500 U/ml and IL-1β (panel 13) doubles NO synthesis in the presence of LPS (central and right bars). Moreover, what is very important, the iNOS induction becomes independent of LPS addition (left bar). No further stimulation is achieved by raising TNF-α to 3,000 U/ml (panel 14).

That TNF-α is not the essential ingredient is corroborated by: (a) omitting it from the mix and raising IL-1β to 100 U/ml, which only slightly decreases stimulation (panel 15), and by (b) supplementing the latter mix with 500 U/ml TNF-α, which although prevents this small reduction does not raise the stimulation further (panel 16), IL-1β can be lowered to 5 ng/ml and IFN-γ to 100 U/ml (panel 17) with little effect on the induction (compare with panels 14 and 16). The possibility of reducing IL-1β to 5 ng/ml without

compromising stimulation is very obvious by comparing panel 18 with the homologous panel 13 where four fold more IL-1β was used.

The time-course of the iNOS induction is shown on **Fig. 4**, where cultures of RP-SMC were treated or not with the standard mix, in the presence or absence of a NOS inhibitor (L-NAME), for periods ranging from 0 to 96 h and nitrites were estimated in the medium. In the absence of an inhibitor there is very little nitrite accumulation even at 96 h (solid squares), but with the standard induction mix, after a 6-10 h lag period of little stimulation, there is a nearly linear synthesis up to 48 h, with a slow-down in this rate after that period (solid circles). That this nitrite release is due to iNOS induction is shown by the up to 85% inhibition obtained with 2 mM L-NAME (solid diamonds).

The slow-down of nitrite synthesis after 48 h in the presence of inducers is not due to a significant increase in iNOS degradation but rather to feed-back inhibition of enzyme activity by excessive product accumulation, as shown on **Fig. 5** (+I). RP SMC were cultured to confluence onto 24-well plates in the absence or presence of inducers, and medium was removed at 48 h and replaced by fresh medium containing inducers or not. When nitrite synthesis is prorated per hour, the value obtained during days 3-5 (hatched bar) is even higher than that occurring in the preceding two days (empty bar). The turnover of the enzyme when the inducers are removed at 48 h and not replaced (**Fig. 5** (-I)) seems to be slow, which should assure the persistence of high iNOS levels for a considerable period after ceasing the induction.

The fact that the observed stimulation of nitrite accumulation in the RPSMC culture medium was due to iNOS induction was confirmed by demonstrating the presence in the induced penile cells of both iNOS mRNA (northern blots), and iNOS protein (western blots). For northern blot analysis of iNOS mRNA expression, mRNA was isolated from RPSMC on two 10 cm dishes by standard guanidium thiocyanate/ CaCl method complemented with two series of phenol/chloroform extractions separated by ethanol precipitation, and polyA+ RNA was isolation by oligo dT chromatography. Northern blots were done with 3-4 ug poly A+ RNA per lane on formaldehyde-denaturing 1% agarose gels, and subsequent transfer to the nylon membranes with 10X SSC, using the Posiblot pressure blotter.

Filters were hybridized as we previously described with a [32-P] labeled specific probe we generated consisting of a 350 bp fragment of iNOS cDNA from RPSMC, designated RPSMC-iNOS350. This fragment was synthesized by us by reverse transcription (RT) of 1 ug of polyA+RNA from RP-SMC induced with LPS/γINF, using antisense and sense primers NO4 and NO3, respectively. These primers are 20-mers designed from the nucleotide sequence of the mouse macrophage iNOS and encompass a 350 hp fragment in the FMN region of this cDNA. This probe was cloned into Invitrogen PCRII vector and subcloned into Promega pGem3z. Automated dideoxysequencing showed this probe to have 92% homology, respectively, to the mouse macrophage iOS cDNAs. After treatment with the NOS probes, northern were re-hybridized with the glyceraldehyde phosphate dehydrogenase (GPDH) probe.

For western blots, other RPSMC dishes submitted to the induction were washed, lysed in a conventional buffer, boiled for 5 min, and the extracts clarified in a microcentrifuge. Aliquots were ran on an SDS electrophoresis minigel with the appropriate molecular weight standards and submitted to a western blot transfer to nitrocellulose membranes. The filters were reacted with a 1/2000 dilution of a

commercial rabbit antiserum against the carboxi terminus of the mouse macrophage iNOS (Affinity Bioreagents). The signal was visualized by a horse radish peroxidase goat anti-rabbit secondary antibody and a commercial ECL (luminol) detection kit.

Fig. 6 shows the northern blot of polyA+ from RPSMC submitted to a time-course of induction, as hybridized with our rat PSMC iNOS probe. The 4.5 kb typical signal is visible on the top part, and the glyceraldehyde phosphate dehydrogenase reference band on the bottom part. **Fig. 7** shows the western blot of an extract obtained by lysis of induced RPSMC and reacted with a commercial iNOS antibody, indicating the expected 125-130 KD iNOS band (lane 2). No signal was obtained with a rat cerebellum extract (lane 1). Lane 3 is empty.

EXAMPLE 2 **Direct Infusion of iNOS Inducers into the Penile Corpora Cavernosa**

This example demonstrates the method for infusing inducers of nitric oxide synthase directly into the penis in a rat.

Male Fischer 344 rats from three age groups designated "adult" (5-month old), "old" (20 month old), and "very old" (28-32 month old) rats, were anesthetized with an intraperitoneal injection of sodium thiopental (pentobarbital) at 50 mg/kg. The definition of these age groups in terms of relative aging corresponds to conventional designations for the Fisher 344 strain of rats (maximal life span approximately 34-36 months; optimum breeding activity 2.5-8 months). The rats were retired breeders whenever possible. Animals were maintained under controlled lighting and were treated according to NIH regulations. The number of animals in each group for each individual experiment is indicated in the corresponding figure.

A transversal suprapubic skin incision (5 mmm) was done to expose the penis angle and its proximal portion. The right corpora cavernosa was then cannulated with a 27-gauge needle attached to a vinyl tubing (Bolab/bb317-85 Arizona) which was connected with an ALZET^{R} osmotic pump (Alza Corporation, Palo Alto, CA). Each pump (100 or 200 ul reservoir volume, as indicated) contained a mix of E. coli lipopolysaccharide (LPS) at 1 ug/ml, recombinant rat interferon-γ) (IFN-γ) at 2,500 U/ml, recombinant human tumor necrosis factor-(TNF-α) at 2,500 U/ml, and recombinant human interleukin-1 β (IL-1 β) at 50 ng/ml. This mix was tested and found effective in the rat corpora cavernosa, and may be used in like methods in human penile tissue (see Example 5), with the substitution of human INF-γ for the rat being preferred.

The needle and the tubing were fixed to the penis peripheral tissues by Mersilene 6-0 suture and the osmotic pump was placed subcutaneously on the rat abdomen. The needle was pierced into the corpora cavernosa, checking its proper delivery into the lacunar spaces by injection or heparinized saline solution and observation of the mechanically induced erection prior to the final connection with the pump. The incision was closed by layer using Dexon^{R} Plus 4-0 suture and the success of the operation was checked all throughout the experiment by observation of a normal recovery and urinary activity. These criteria were completed by verifying the absence of peripheral hematomas or inflammation when the pump was removed. Each pump delivered the inducer mix by osmotic pressure through the catheter directly into the corpora cavernosa, either at 1 ul/hr during 3 days (pump 1003D) or at 0.5 ul/hr during 14 days

(pump 2002).

In certain cases pump 1003D was removed from the anesthetized rat at the third day, and a new pump with fresh solution was instilled in the abdomen and connected to the catheter as above. The treatment proceeding until all the content of the reservoir was expelled, for an additional 3 days period.

EXAMPLE 3 **Measurement of the Improvement of Erectile Response**

This example demonstrates the method for measuring the improvement in erectile response caused by treatment with inducers of nitric oxide synthase.

At the completion of each treatment rats were anesthetized as in **Example 1** and the erectile response was measured by a modification of a published procedure. Briefly, the cavernosal nerve was surgically exposed and stimulated with a square pulse stimulator connected to a platinum bipolar electrode positioned on the nerve. Through a needle inserted into the cavernosa, the intracavernosal pressure was recorded with a pressure transducer connected to a recorder that was calibrated with a manometer in order to express the response in mm of mercury. The animals were reinjected every 45 min with 35 mg/kg of ketamine for the whole duration of the experiment (about 2-3 hours).

Each rat was submitted to the following sequential treatments done in duplicate: a) electrical field stimulation (EFS) at a frequency of 15 Hz for pulses of 30 sec, separated for 5 min intervals, for the voltage response curve, at 15, 10, 2.5, 5 and 10 volts, in this order; b) EFS at 10 volts with the nitric oxide synthase (NOS) inhibitor N -nitro-L-arginine methy ester (L-NAME) at 2 mg/kg, at a single dose per animal, recording 30 min. after injection. In some animals, the systemic blood pressure was measured at the beginning of the experiment, by intrafemoral cannulation and recording as above. Means and standard deviations were determined for the intracavernosal pressure values, and the statistical significance was obtained by the paired Student's t test.

Fig. 8 shows that there is a significant increase of the erectile response to EFS measured by the maximal intracavernosal pressure at 10 volts in adult and old rats, but a non-significant stimulation in very old rats, treated for three days with the 100 ul pumps (open symbols), as compared with rats of the same ages not submitted to this treatment. At the 2 volt threshold the stimulation of erectile response is significant in all groups. Treatment of the old rats displaying erectile dysfunction with the iNOS inducer mix turns them into better respondents than the younger animals (adult group).

This considerable enhancement of penile erection found even in non-aged (adult) rats is not accompanied by undesirable side effects. The rats look healthy and alert, their systemic blood pressure remains normal, and there is no indication of priapism. This suggests that whatever the mechanism of penile erection enhancement, it remains under physiological control which is released by the EFS stimulus. While we do not wish to be bound by theory, we believe the EFS stimulus elicits a nerve transmission process in the penis similar to one or more processes occurring during sexual stimulation, or responsive to sexual stimulus.

Fig. 9 shows that the stimulation of penile erection in the treated rats is dependent on the NOS cascade, since it is inhibited by sub-optimal doses of L-NAME. The fraction of maximal intracavernosal pressure remaining after L-NAME treatment was established and compared with equivalent values in

untreated rats previously obtained by us in a separate study. The comparative values in the treated and untreated rats were: 39% vs 62% (adult), 18% vs 30% (old), and 28% vs 23% (very old), in the treated vs untreated rats. This indicates that in the adult and old animals the dependence on the NOS cascade upon treatment becomes even higher than in the absence of NOS inducers and in the senescent remains the same.

The efficacy of the short-term treatment (3 days) over longer treatments where the inducer mix remains in the pump for 14 days, with the possibility of inactivation of some of the biological constituents, is shown in the experiment depicted on **Fig. 10.** This illustrates the effects on the erectile response to EFS after the 14 days treatment with the same inducers used in **Fig. 8.** Two groups of rats are apparent in each age group: a) high respondents (solid symbols), with up to 50% and 300% stimulation of the intracavernosal pressure at 10 and 2 volts, respectively, above the respective untreated rats (shown on **Fig. 8**); b) non-respondents (open symbols), with values similar or below the untreated rats.

The success of a treatment with iNOS inducers can be judged by the sensitivity to a sub-optimal dose of L-NAME, as shown on **Fig. 11.** Even more evident than in the case of the experiment depicted on **Fig. 9**, L-NAME inhibited by over 95% the erectile response of the respondent animals in the three age groups, whereas in the non-respondent rats the inhibition was normal (as in the untreated rats).

These results show that iNOS inducers administered locally to the rat penis (or introduced in the tissue) effectively stimulate to a considerable extent the erectile response to EFS in all three age groups, and even more substantially in old rats.

EXAMPLE 4 **Measurement of Increased NOS Activity After Inducer Treatment**

This example demonstrates that the improvement in erectile response is accompanied by the stimulation of nitric oxide synthase.

Six adult rats were implanted with 100 µl pumps for 3 days, and then the pumps were replaced by similar ones with fresh solution as indicated in **Example 2.** Three animals had the pumps containing the inducer mix and three had saline as a control. At the end of the experiment the animals were anesthetized as indicated above and the penis (including the bulb), liver, and in some animals the cerebellum were surgically removed. The penile head and skin were excised and the organs were stored under liquid nitrogen.

NOS activity was determined from tissue homogenates from two treated and two control rats, not subjected to EFS in order to avoid the interference with residual L-NAME from the in vivo experiments and EFS-induced changes in NOS activity. Homogenates were prepared from each individual organ (approximately 300-400 mg), in 4 volumes of cold medium containing 0.32M sucrose/20 mM Hepes pH 7.2/0.5 mM EDTA/1 mM DTT, and protease inhibitors (3 µM leupeptin, 1 µM pepstatin A, 1mM phenylmethyl sulfonylfluoride. The cytosol and particulate fractions were separated by centrifugation at 12,500 g for 60 min, and the particulate fraction was resuspended in an equal volume of medium. The cytosol fraction was passed through Dowex AG50WX-8 (Na^+) resin to remove endogenous arginine and 50 ul aliquots were incubated in triplicate for 45 min at 37 C as indicated previously, in the presence of 2

μCi/ml resin-purified (3-H) L-arginine 100 μM L-Arginine, with or without L-NAME (2 mM), 0.3 mM aminoguanidine or EGTA (5 mM). After eliminating the residual (3-H)L-arginine through the resin, (3-H) citrulline was counted in the trichloroacetic acid ether-extracted supernatant. All values were corrected by the radioactivity eluted in time zero incubations.

Fig. 12 compares the NOS activity in the penile cytosol of untreated (control) rats, column labeled "C", with the iNOS inducer- treated rats, column labeled T. The 58% increase in NOS activity is clear. As **Fig. 13** shows, this activity is 60-70% inhibited by L-NAME and not affected by aminoguanidine, AG. Since AG is a preferential inhibitor of macrophage iNOS as compared to nNOS, this evidence leads us to believe the iNOS induced in vivo is not the same as macrophage iNOS.

The effect of iNOS inducers in increasing penile NOS was confirmed by histochemical detection of NADPH diaphorase activity. One penis from a treated adult rat and one penis from a control adult rat were embedded in OCT compound frozen at -70°C, cut in 15 μm sections with a cryostat, and submitted to NAPDH diaphorase staining with tetrazolium blue. This activity is recognized as co-localizing with NOS in all tissues so far studied, including the rat penis.

Fig. 14 (all at 100 X magnification) shows the comparison of frozen sections from untreated rat corpora cavernosa from 20 month old animals (panel A), from induced corpora cavernosa from the same age group (panel B), and from the rat cerebellum (panel C). It is obvious that while the blue dye is restricted to small areas in the untreated penis, it is more intense and spread over larger areas in the corpora cavernosa from rats treated with the iNOS inducers. The cerebellum shows a wide area throughout most of the tissue section.

A confirmation of the NADPH diaphorase staining was obtained by immunocytochemistry with a commercial rabbit polyclonal antibody against mouse macrophage iNOS and biotinylated goat antirabbit IgG, using horse radish peroxidase and DAB staining (**Fig. 15**, all at 100 X magnification)). Panel A shows little staining of untreated rat corpora cavernosa with control rabbit serum (1/1000), whereas the antiserum (1/1000) stains some areas in a more intense brown color (panel B). Sections from the treated corpora cavernosa (panel C) have a darker and more difuse staining, as in the case of NADPH diaphorase. The immunocytochemical procedure allows a more specific recognition of the iNOS isozyme than the NADPH diaphorase technique (NOS in general), and suggests that iNOS is expressed in the penis from old rats even in the absence of external induction. However, induction stimulates iNOS expression as expected.

A confirmation of iNOS induction in the penis of 20-month old rats treated with iNOS inducers was obtained by analyzing the penile cytosol from two treated and two control animals by Western Blot assay. Protein was estimated by a Lowry procedure and 80 μg of cytosol protein were run on 7.5% polyacrylamide minigels for 1 h and transferred to nylon membranes by electroblotting. The iNOS 130 kD band was detected as in the previous example, using 1 h exposure for the autoradiography.

Fig. 16 (bottom) shows a faint but distinctive iNOS signal in the penile cytosol (CC) from the untreated rats (Ut, first two lanes), which confirms the immunocytochemical results indicating a degree of physiological expression of iNOS in the rat penis, at least in the aged animals. As observed by immunocytochemistry, the iNOS signal is considerably enhanced in the penile cytosol from the pump-

treated rats (Tr), proving that the in vivo induction was successful. No iNOS band is seen in the lanes corresponding to the two negative controls (cerebellum (Ce), and untreated RPSMC), whereas a clear signal occurs in the induced RPSMC cytosol.

The membrane was then stripped from the iNOS signal and reacted again with a rabbit antibody against the carboxy terminus of the human brain neuronal NOS (nNOS) (Transduction Laboratories). The corresponding 160 kD species was detected as above, as the top band in a doublet. **Fig. 16** (top) shows the presence of nNOS in the four penile samples and particularly in the cerebellum cytosol (positive control), and its absence in both untreated and induced RPSMC. This in turn confirms the specificity of the detection with the iNOS antibody.

The data provided in Examples 3 and 4 demonstrate the process of the invention of inducing iNOS in the rat penis by a direct delivery of the inducer mix to the penis and the resulting stimulation of the erectile response in three age groups without undesirable side-effects.

EXAMPLE 5 Ex vivo detection of iNOS induction in the rat and human corpora cavernosa

In order to confirm the capacity of the iNOS inducer mix to elicit iNOS synthesis by direct effect on the rat penis, it is important to demonstrate that iNOS induction can occur in the penile tissue excised from the animal. Similarly, since the ultimate goal is human therapy, it is essential to show that this process can also occur in the human penis. This example shows that iNOS can also be induced by inducer mixes in the corpora cavernosa tissue.

The skin-denuded bulb and shaft penile regions were excised from three rats, cut in very small pieces, washed several times, and incubated in 24-well plates (50-80 mg/well) in 0.5 ml MEM/10% fetal calf serum. Each penis was divided in four wells, and two of the wells received the standard binary incubation mix (10 ug/ml LPS and 250 U/ml rat γ-interferon). Incubation proceeded for 48 h and nitrites were estimated in the incubation medium. The tissue pieces were washed, homogenized, and the cytosol obtained as in the case of RPSMC in the previous examples. Western blot analysis of iNOS protein was done as above, except that another commercial antibody (Upstate) was used, directed against the whole mouse macrophage iNOS protein. This experiment was repeated three times.

For the analysis of the induction in the human penis, pieces of corpora cavernosa were obtained with informed consent as discarded material from three patients undergoing implants of penile prosthesis. The incubation and analysis were carried as above separately in the tissue from each patient, except for the following: a) the penile pieces were submitted overnight to a serum depletion shock in MEM/0.1% human serum, and then the incubation was continued in the same medium for five days; b) the inducer mix was 10 ug/ml LPS, 500 U/ml human γ-INF, 200 U/ml human TNFα, and 4 ng/ml human IL1β.

Fig. 17 top shows the results obtained with the rat corpora cavernosa (RCC) in rats 1-3, with (+) or without (-) induction. An intense 130 kD iNOS band was detectable by Western Blot in the cytosol from the three penises incubated with the inducer mix. The position of the iNOS band was confirmed with cytosol from induced mouse macrophages (IMM, last lane). In two cases (rats 1 and 3), no iNOS band was visible in the absence of inducer, but in one rat (rat 2) there was a substantial endogenous iNOS synthesis, although lower than in the induced samples. Nitrite production was evident in all cases, but

at low level (44-65 uM in the induced samples). No clear differenc in NO synthesis was observed between the + and -lanes, and ther was no correlation between the intensity of the iNOS band and nitrite production. This contrasts to what occurs with induced and not induced RPSMC (see above). Similar results were obtained in the replicate experiments (not shown).

Fig. 17 bottom shows the results with human penis corpora cavernosa (HPCC). A very intense iNOS band was detected in the third lane of the Western Blot, corresponding to the induced pieces of HPCC tissue. The positive controls were the cytosols from induced RPSMC (lane 4) and mouse macrophages (IMM, lane 5). No iNOS was seen in pieces of the same tissue prior to incubation (lane 1), or after incubation in the absence of inducers (lane 2). Similar results were obtained in the other two replicate experiments (not shown). As in the case of the rat corpora cavernosa, the poor amount of nitrites synthesized by the human corpora cavernosa slices (58 μM here, only 20-30 μM in the other patients) does not correlate well with the intense iNOS band observed.

These experiments prove conclusively that: a) iNOS can be induced ex vivo in the rat corpora cavernosa, confirming the results obtained in vivo; b) iNOS can also be detected at low levels in some rats in the absence of added inducers, as previously observed in vivo; c) the induced iNOS appears to be subject to a tight control of enzyme activity (inhibition), because nitrite synthesis is very poor and most likely arises from nNOS activity; d) essentially the same process occurs in the human corpora cavernosa.

The overall conclusion from this example is that iNOS protein or cDNA introduced in the corpora cavernosa by pharmacological means (mRNA induction by inducer mixes, in vivo or ex vivo gene therapy, protein administration, etc) will remain inhibited by a physiological control and will not produce NO until the control is released, e.g., by one or more natural sexual stimulants. This confirms applicability of the process of the invention as therapy for impotence by not allowing an undesired permanent vasodilation to occur in the treated corpora cavernosa (priapism). In addition, it explains the absence of priapism in the treatment of rats both with inducers in pumps (Example 3) and with cDNA gene therapy (Example 7). The release of control occurs in our experiments in the rat by the electrical stimulation of the cavernosal nerve (EFS), which we believe mimics the nerve conduction resulting from sexual stimulus, and is expected to involve Ca2+ mobilization or other mechanisms of NOS activation. This EFS activation has been shown by us to occur at least in the case of castrated rats.

EXAMPLE 6 **Cloning of penile specific iNOS cDNA and demonstration of its ex vivo expression for subsequent inoculation of transfected cells or tissue explants into the rat and human corpora cavernosa**

In order to carry iNOS gene therapy it is essential to have constructs with the complete iNOS coding region, and it is important to determine whether the penile iNOS nucleotide and amino acid sequences in these constructs differ from those reported in the literature for other iNOS. A distinctive penile iNOS cDNA may derive from an iNOS gene, or genes, expressed preferentially in the penis that may be subject to differential expression regulation. The enzyme activity of its product (iNOS protein) may also be controlled specifically in the penile tissue. This example shows the construction and use of a rat iNOS cDNA construct to express constitutively the iNOS protein in RPSMC.

Two approaches are presented for making the iNOS constructs. The first one was focused on the rat penis iNOS from induced RPSMC, because it allows for gene therapy experiments to be first tested in this animal. The other approach was on human penile iNOS, because it is the product to be used for therapy of human impotence based on the experiments in the rat.

Total RNA was isolated from confluent RPSMC incubated in 150 cm2+ flasks with the LPS/INF inducer mix for 48 h, and the polyA+RNA was prepared by conventional procedures. 15 μg of this RNA were used for the construction of an induced-RPSMC cDNA library into the σZap vector (UniZap XR, Stratagene). This library was plated on 150 cm^2 Petri dishes, cultured, and the lysed plaques were transferred in duplicate (replica plating) to nylon membranes. Hybridization was carried out with the RPSMC-iNOS350 labeled with 32-P. After a primary, secondary and tertiary screening, four clones were selected for sequencing using the dideoxy procedure in an automatic Sequenator. Each sequencing was extended to the 3' direction with the help of successive sequencing primers synthesized on the basis of each previous sequence. Antisense primers allowed sequencing of complementary sequences.

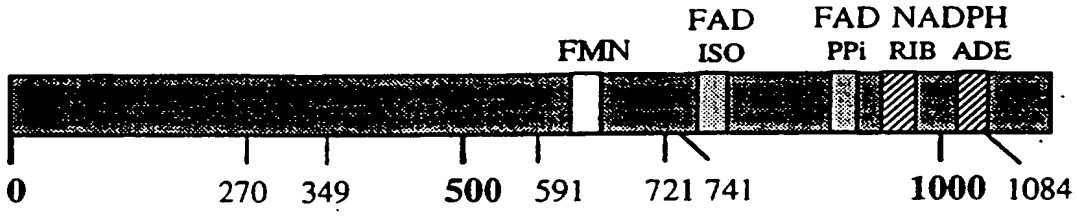
Because of library preparation artifacts only 1.1 kb could be sequenced properly, and the four clones were overlapping to a large extent. Sequencing of 5' and 3' regions was obtained by RT/PCR from the RNA used for constructing the library, as depicted on **Fig. 18**. The four clones are indicated above the schematic representation of the iNOS cDNA, as TSC8, TSC5, TSC2, and C5y, and the three PCR products 1/2, 5/6, and 7/8, are also shown. The arrows below the iNOS outline show the length and direction of each individual sequencing.

In order to obtain more representative clones and complete the sequencing, a second cDNA library was obtained from a new polyA+ RNA preparation from induced RPSMC. The library was screened with a mixture of the probes depicted on Fig. 18. Four clones were selected and sequenced as above, as represented on **Fig. 19**. Only clones 1 and 5, used for the final construct are depicted. Clones 2 and 3 overlap with 5 and slightly extend to the 5' region. The arrows below the iNOS outline show the length and direction of each individual sequencing.

The overlapping fragments from both cDNA libraries allowed to edit the sequencing errors or ambiguities, and a consensus sequence was finally obtained for RPSMC iNOS (see Appendix A for the nucleotide sequence listing for the RPSMC iNOS cDNA). Table I below compiles the nucleotide and amino acid differences found with the sequences of rat vascular iNOS from two different groups, and shows six amino acid differences when matched separately to each one of the reference sequences. The number of amino acid residues which differ from both reference sequences simultaneously is two (amino acids 270 and 591) in regions placed upstream from the enzyme active groups. The existence of two clear variations suggests that the iNOS RPSMC sequence arises from a different gene than the rat vascular sequence. This conclusion is supported by the recent discovery of multiple iNOS gene sequences in the human and primate genome, even in separate chromosomes (14 and 17). However, polymorphisms cannot be entirely discarded.

TABLE I

Different amino acids found in the RPSMC iNOS as compared with the rat vascular SMC iNOS



Change No.	AA POS. No.	RPSMC THIS WORK		RAT VASCULAR SMC REF # 1		RAT VASCULAR SMC REF # 2	
		codon	AA	codon	AA	codon	AA
1	72	CAU	HIS	CAU	HIS	UAU	TYR
2	201	AUU	ILE	AUU	ILE	AUC	ILE
3**	270	ACC	THR	CCC	PRO	CCC	PRO
4	348	GCC	ALA	GCC	ALA	CCC	PRO
5*	349	GUG	VAL	GCG	ALA	GUG	VAL
6	423	UUU	PHE	UUU	PHE	UUC	PHE
7**	591	GGC	GLY	GTC	VAL	GTC	VAL
8	679	GAG	GLU	GAG	GLU	GUG	VAL
9	680	ACG	THR	ACG	THR	CCG	PRO
10	683	GUU	VAL	GUU	VAL	GUC	VAL
11*	721	CUC	LEU	CCC	PRO	CUC	LEU
12*	741	CUG	LEU	CCG	PRO	CUG	LEU
13*	1084	AUG	MET	AUC	ILE	AUG	MET

Ref #1 :cDNA cloning and expression of iNOS from rat vascular smooth muscle cells. Biochem. Byophys. Acta., Geng, Y.J. et al.. 1994.

Ref # 2: Cloning of iNOS in rat vascular smooth muscle cells. Biochem. Byophys. Resear. Com., Nunokawa, Y. et al.,1993.

*Matches ref.# 2, but not ref.# 1

No * matches ref.#1 but not ref.# 2

**Do not match neither ref.# 1 or ref.# 2.

Underlined: data similar to our work

Two of the clones from the second library (1 and 5) were used to make a construct of the RPSMC iNOS coding region in vector pBlueScript SK (+/-) (Stratagene). Sticky ends were created in clone 1 with restriction enzymes KpnI and NheI and in clone 5 with NheI and XhoI. Both fragments were ligated and the dimer separated by agarose electrophoresis and inserted into the vector that had been restricted with KpnI and XhoI. The ligation mix was used to transform E. coli and the plasmid isolated, purified, analyzed, and designated pBS RPiNOS. The entire iNOS coding region was then cleaved from pBS RPiNOS using KpnI and NotI and cloned into those cloning sites in the eukaryotic expression vector pcDNA3 (Invitrogen). This vector contains the CMV promoter to direct the transcription of the cloned gene, and SV40-driven neomycin gene conferring resistance to the antibiotic G418. The new construct was designated pcDNA3 RPiNOS and sequenced for 150 bp at the 5' end, confirming the presence of iNOS (see Appendix B for the RPSMC iNOS amino acid sequence).

The construct was then used to stably transform RPSMC in vitro in order to make the cells to constitutively express the iNOS protein in the absence of iNOS inducers. pBS RPiNOS was mixed in a 1:8 ratio (ug/ul) with lipofectin (GIBCO) and added (2 μg/well) to 1 ml serum-deficient DMEM medium onto 30% confluent RPSMC growing on 35 mm plates. After 5 h DMEM containing 10% fetal calf serum was added and the incubation was allowed to proceed for 24 h. At this time the medium was replaced by fresh one and the incubation continued for 48 h. Medium was again replaced and the culture was continued in medium containing 300 μg/ml G418 to select the transformed cells containing the plasmid. Multiple cell clones were isolated after 3-4 weeks of growth and propagated. 16 clones were chosen and tested for nitrite production in the absence of inducers. Several clones released nitrites on 4 day incubations in amounts above the basal 10 μM concentrations for non-transfected cells, to levels of up to 45-50 μM. This clearly shows an unexpectedly high expression of active iNOS in the transfected cells.

EXAMPLE 7 **Partial sequencing of the human penis INOS cDNA for the therapy of human impotence**

In order to transfer to the human the iNOS gene therapy strategy developed in the rat model, the human penile iNOS must be sequenced to determine whether it is the same species or a different one from the human hepatocyte cDNA, and cloned into an adequate expression vector. The latter allows experiments in HPSMC and human corpora cavernosa slices to be conducted prior to human clinical trials. This example shows the relevant partial sequence (60% of the coding region) of the human penile smooth muscle iNOS cDNA.

Specimens of surgically excised corpora cavernosa (less than .5 g per patient) were obtained from impotent patients undergoing penile prosthesis implantation, and placed in (DMEM) on ice until transport to the laboratory. All procedures were approved by an institutional human subjects committee and carried out under informed consent protocols. The tissue was sliced in very small fragments and used upon 2-6 h of excision for starting up cultures of penile smooth muscle cells (HPSMC) in DMEM with 20% fetal bovine serum using the standard explant method on Primaria flasks. Other tissue pieces were used for direct treatments (see above) without prior smooth muscle cells isolation. Once cells reached confluence they were trypsinized, transferred to DMEM with 10% fetal bovine serum, and replicated for up to 25

passages. These cultures were shown to contain only smooth muscle cells by visual inspection of their morphological features and by immunocytochemical staining for muscle actin. Cultures were designated HPSMC ("human penis smooth muscle cells"), and each one originating from an individual patient was given an identification number.

In order to determine the most adequate conditions to induce the synthesis of iNOS, HPSMC were transferred onto 24-well plates and allowed to reach confluence. Homologous cultures of RPSMC from passages 4-14 were used as reference. A series of inducers were added at various concentrations, and NO production was measured at 24 and 48 hours by determining the release of nitrites to the medium, using the standard Griess reaction.

In method 1, HPSMC from three different patients at passages 4-14 were treated directly in th presence of DMEM/10% fetal bovine serum, whereas in method 2, HPSMC from two of these patients (passage 12) were switched to DMEM with 0.1% of bovine or human serum, maintained for 24 hours, and finally incubated for another 24 hours in the fresh corresponding medium containing the different inducers. The latter procedure worked satisfactorily for incubations of human corpora cavernosa

HPSMC showed very little nitrite production by treatments that elicited a considerable increase in nitrite synthesis in RPSMC, except for a few instances where there was an occasional elevation of iNOS activity in HPSMC. In one of the latter cases poly A+ RNA was isolated by a conventional procedure from HPSMC that were grown onto 10 cm^2 dishes and induced for 48 hours with method 2 with a mix containing E. coli lipopolysaccharide (LPS) at 1 μg/ml, recombinant human interferon-gamma (IFN-γ) at 500 U/ml, recombinant human tumor necrosis factor-α (TNF-α) at 200 U/ml, and recombinant human interleukin-1β (IL-1β) at 4 ng/ml. Due to the relatively low iNOS induction in HPSMC, this cDNA library is not the preferred method.

The preferred method was changed from the cDNA library approach applied to induced RPSMC, to one based on reverse transcription (RT) of the iNOS mRNA combined with the amplification by polymerase chain reaction (PCR) of the resulting cDNA. A series of 18 twentymer primers were synthesized by a standard procedure using the published sequence of the human hepatocyte iNOS cDNA, for RT/PCR and DNA sequencing reactions. They were chosen so that each one of the sense primers (except No. 1) is 100-150 bp upstream of the immediately preceding antisense primer, encompassing a unique restriction site in each of the different fragments (0.35-0.95 kb). This facilitates the subsequent cloning of the amplified fragments.

Aliquots of 0.5 μg of polyA+ RNA from method 1 in Example 1 were reverse transcribed by a standard procedure using MMLV reverse transcriptase (100 U) in the presence of each one of the antisense oligonucleotide primers (0.5 μM). An aliquot of 1/5 of the RT mix was submitted to PCR in the presence of the sense and antisense primers (0.25 μM) in a series of 35 cycles at 94 C (45 sec), 60 C (30 sec), and 72 C (90 sec), using the conventional "hot start" method. An aliquot of 1/7 of the PCR mix was analyzed by gel electrophoresis and the size of the bands determined by comparison against an adequate standard. The remainder of the PCR mix was submitted to the same procedure and the fragments were eluted and purified by conventional methods (Millipore column).

Each fragment was sequenced in an automatic sequencer using both the respective sense and

antisense primers. The location of the HPSMC iNOS cDNA that has been sequenced is depicted on **Fig. 20** as shaded areas, and extends for 2.13 kb out of 3.63 kb. This encompasses the protein kinase, calmodulin, FMN, and FAD binding sites. Two anti-human hepatocyte iNOS antibodies designated Ab1 and Ab 2 were prepared in rabbits against synthetic small peptides (15 amino acids each) in those regions.

Fig. 21 shows the primers utilized for generating the RT/PCR fragments from HPSMC that were sequenced. **Fig. 22** shows that the homologies between the HPSMC iNOS nucleotide sequences and the respective human hepatocyte iNOS varies from 88% to 99% according to the region. The homology of HPSMC iNOS to the rat aorta iNOS is only 81-82%. Even considering ambiguities and sequencing errors, this suggests that the HPSMC sequences so far obtained belong to a different cDNA than that for the human hepatocyte iNOS, in agreement to the situation obtained with RPSMC iNOS. However, whether or not RPSMC and HPSMC iNOS cDNAs are coded by different gene(s) than the rat and human iNOS gene(s) so far discovered does not affect the use of the iNOS gene therapy or iNOS protein therapy procedures of this invention.

This example shows the procedure used to clone the penile iNOS from the corpora cavernosa smooth muscle and the sequence of a penile-specific new iNOS isoform. The corresponding construct has been expressed in rat penile cells that are able to constitutively synthesize NO. Accordingly, one method of this invention comprises implanting iNOS stably transfected penile cells or pieces of corpora cavernosa into the penis of men with erectile dysfunction, in order to increase NO synthesis upon sexual stimulation and improve the erectile response. In addition, penile iNOS from the human corpora cavernosa smooth muscle has been partially sequenced and is believed to be a distinct penile isoform. (See Appendix C, and the corresponding cDNA sequence Appendix D.)

EXAMPLE 8 **Correction of erectile dysfunction the aged rat by gene therapy of the corpora cavernosa with rat penile iNOS cDNA**

In order to prove that iNOS can ameliorate the erectile dysfunction in aged rats it is necessary to administer the construct in vivo, measure the erectile response of the treated animals, and show that iNOS is expressed in the corpora cavernosa. In addition, it is important to assess the possible side effects both at the penile and systemic levels. This example shows that gene therapy of erectile dysfunction in a rat model using the RPSMC iNOS cDNA construct significantly and unexpectedly improves erectile response.

Seven 20 month-old rats and one 5 month-old rat castrated for one week were selected for this experiment. Aseptically, using a small Pfannesnstiel incision in anesthetized rats the penis was exposed. Blunt dissection of the length of the penis was achieved. Using a 2-0 silk and clamp, the penis was constricted at its base. Three of the intact rats and the only castrated rat received a 100 ul mix of a freshly prepared mix of 10 μg pcDNA3 RPiNOS and 80 μl lipofectin (1:8 ratio) injected into the corpora with a 25 gauge needle. This position was maintained for 2 min. The needle was then removed and the incision closed with interrupted 2-0 silk sutures. The remainder four intact aged rats (controls) were treated with the lipofectin only, with the procedure described above. Five days later the EFS assay was applied to measure the erectile response, while simultaneously recording the mean arterial pressure using a computer data acquisition and analysis system.

Two of the intact rats (#1 and #2) had an unexpectedly dramatic and significant improvement of the maximum intracavernosal pressure (MIP) at 10 volts EFS, reaching 105 and 100 mm Hg in 2-3 stimulations (**Fig. 23**). One of the control rats (#4) showed an MIP of 45 mm Hg, and the other two (not shown) were 50 and 58 mm Hg. All the control rats were at or below the mean previously established for 20 month-old rats (see Example 3) of 58 mm Hg. The stimulation of the erectile response was even above the mean previously obtained for adult rats (77 mm Hg). Erection was completely abolished by injecting systemically the NOS inhibitor L-NAME at 2.5 mg/kg, thus clearly establishing that the response is NO-dependent. The erectile responses of a third aged treated rat (#3) and the castrated adult rat (not shown) were not improved by the treatment (60 mm Hg). Castration is known to affect the bulbocavernosus/ ischiocavernosus penile muscles in the rat, which participate in penile erection in rats and men. Therefore, the castration-related erectile dysfunction is currently considered 'a priori' to be probably refractory to iNOS therapy.

No side effects were apparent in the treated rats. The mean arterial pressure remained as in the control rats and responded the same way to L-NAME (slight increase). If a systemic effect would have occurred, it should have led to hypertension. Assessed visually, no priapism, penile damage or impairment to the rat alertness, was evident.

The constitutive iNOS expression in the penis from treated rats could be detected by Western Blot analysis of the penile cytosol. **Fig. 24** shows a very distinctive iNOS band in the corpora cavernosa of rat #1, a fainter one in rat #2, and none in rats #3 and 4#, which is expected from the results of the erectile response. Three of the controls (#2,#3,#4) had little or no iNOS expression, also agreeing with the erectile response. However, one control (#1) had an apparently spurious high iNOS not consistent with the erectile response.

Another group of four 20 month-old rats were injected with 1/4 of the dose of construct/liposome complex given above and the erectile response to EFS was measured 11 days later. There was a dramatic increase of the MIP to a mean+/- standard error of 107+/-10 mm Hg, a value much higher than for untreated 20 month-old rats (57+/-10 mm Hg), and higher even than the one for adult rats (79+/-8mm Hg). The mean arterial pressure in the rats submitted to gene therapy was 133+/-9 mm Hg, and the MIP/MAP ratio was 0.80+/-0.03.

Example 8 shows that a single injection of our construct given directly in the corpora cavernosa was able in two aged rats out of the three tested to improve dramatically the EFS erectile response 5 days after the injection, and to elicit penile iNOS expression, without causing side effects. The response, after a much longer period to allow for a higher iNOS expression, and using a lower dose to minimize side-effects, was even better and more reproducible. This is compelling evidence that gene therapy of impotence may be performed in the patient by self-injection directly in the corpora cavernosa. However, the patient only needs to self-administer these injections only periodically (at a frequency proportional to the stability of NOS expression). This may be done in privacy for a long-term effect, not immediately prior to the sexual act, as with vasoactive drugs.

EXAMPLE 9 **Preparation of recombinant rat penile iNOS enzyme for inoculation into the corpora cavernosa**

In order to obtain an immediate and medium-lasting effect on NO synthesis in the penis and the subsequent erection which is useful in less severe dysfunctional cases, the invention includes a procedure employing inoculation of the recombinant penile iNOS protein directly into the corpora cavernosa. This "foreign" iNOS enzyme becomes active only when sexual stimulus triggers the initial erectile response. The same result is expected to occur with endogenous iNOS synthesized from induced iNOS mRNA or from transfected recombinant iNOS. This example shows methods of production of large amounts of penile iNOS protein for use in erectile dysfunction therapy.

To produce in vitro large amounts of RPSMC iNOS protein, the full length rat iNOS gene cloned by us into the pBluescript SK- plasmid (pBS RPiNOS) was moved into the baculovirus transfer plasmid pVL1393 by a multi-step approach.

First, the 5' region of the RPINOS gene was removed by PCR. Efficient expression of genes from the polyhedron promoter on pVL1393 requires that the ATG translational start sequence be within 80 base pairs the polyhedron promoter. The cloned RPINOS gene has a 142 bp 5' untranslated region. We chose to remove this region by performing PCR on the RPINOS gene using primers matching the polyhedron promoter-BamHI restriction site and an internal region of RPINOS spanning a BsaAI restriction site. This 254 bp PCR fragment was next cloned into the pCRII cloning vector by the TA cloning technique. The plasmid pCRII-BamHI-BsaAI was cut with BamHI and BsaAI restriction enzymes and the BamHI-BsaAI fragment was used later for a ligation reaction.

The second step was to isolate the 3' region of RPINOS and the baculovirus transfer vector, pVL1393. Plasmid pBSRPINOS was cut with BsaAI and NotI to liberate a 3.8 KB fragment that was subsequently purified for cloning purposes. pVL1393 was cut with BamHI and NotI restriction enzymes and purified. The fragments were then linked together by first ligating the 254 bp BamHI-BsaAI fragment with the 3.8 KB RPINOS 3' region fragment. This product was isolated by gel electrophoresis and subsequently ligated to pVL1393 (BamHI and NotI cut). The composite plasmid, pVLRPiNOS, was transformed into E. coli and the correct ligation product was identified by colony hybridization using separately the 254 bp BamHI-BsaAI fragment and an internal RPINOS gene fragment. The cloning was further corroborated by restriction digestion and DNA sequencing of the RPiNOS ATG region.

For expression of RPiNOS in insect cells, the gene must be moved into the baculovirus genome by homologous recombination. This was done by co-infection of Sf9 insect cells with the transfer plasmid pVLRPiNOS and a deletion version of baculovirus AcNPV (Baculogold, sold by Pharmigen). The modified virus allows positive selection for recombinants. Following co-transfection, 8 individual virions were purified by a plaque assay. Four of these pure isolates were amplified twice to obtain high titer stocks.

Viral stocks were analyzed for expression of iNOS by a variety of methods. First, PCR on viral supernatants and infected cell cytosols to identify which are correctly recombined. Second, Western Blotting for the level of production of iNOS. A high level baculovirus producer of iNOS infected a large culture of Sf9 cells grown in spinner flasks. The iNOS is purified by 2' 5' ADP-ribose affinity

chromatography, which yields an approximately 95% purified protein, which is useful for in vivo gene therapy experiments in the rat. The corresponding human penile iNOS, produced by the same method is useful in in vivo gene therapy in human patients.

EXAMPLE 10 **Rat models for testing the efficacy of NOS biological agents and proposed routes of administration in humans**

In order to demonstrate the usefulness of iNOS therapy for different types of erectile dysfunction, it is important to determine in each case whether endogenous penile NOS is actually decreased and at what level of NOS expression the impairment occurs. The rat model of erectile dysfunction allows for the systematic determination of erectile function and penile NOS. This example shows that erectile dysfunction in the rat model is ordinarily accompanied by a decrease in penile NOS.

For the study of short-term effects inducing the decrease of serum androgens or interfering with their tissue targets, adult (5 month old) Fischer 344 rats were castrated and treated for one week with the compounds indicated on Table II below, or submitted to adrenalectomy. Castration blocks the gonadal production of serum testosterone (T), and adrenalectomy suppresses androgens made by the adrenals plus other cortosteroids. Flutamide is an antiandrogen that eliminates androgen effects at the tissue level. Finasteride is an inhibitor of the conversion of T into DHT. Other groups of adult rats were left intact and submitted to hypophysectomy, adrenalectomy, or treatment with an inhibitor of the hypothalamic/pituitary axis (GnRH antagonist). The latter regulates the production of T by the testis. Untreated intact and castrated rats were used as controls.

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TABLE II

IMPOTENCE RISK FACTORS ASSOCIATED WITH A DECREASE OF PENILE NITRIC OXIDE SYNTHASE IN THE RAT

CONDITION OR TREATMENT	REF.	EFS RESPONSE	PENILE REFLEXES	NOS ACTIVITY	NOS CONTENT
A) SHORT-TERM HYPOGONADISM #					
CASTRATION ALONE	1	-		-	NC
" & FLUTAMIDE	2	--		-	NC/-
" & T & FINASTERIDE	1	-		-	
" & ESTRADIOL	2	--		-	
HYPOPHYSECTOMY	2	--		--	NC/-
GnRHA	2	-		-	NC
ADRENALECTOMY	3	-		-	NC
CASTRATION & ADRENALECTOMY	3	--	--	-	
B) LONG-TERM CONDITIONS					
AGING, VERY OLD	# 4	--		--	
DIABETES TYPE I	# 5,6	NC	--	--	-
DIABETES TYPE II	# 5,6	NC	--	--	-
PASSIVE SMOKING	7	NC		--	-
" " & AGING, OLD	7	NC		--	-

Changes as compared to respective control rats. NC: no change; NC/-: non-significant 20-30% decrease; -: 40-50% decrease; --: more than 60% decrease.
Mean arterial pressure normal, except in ".
Serum T lower in #.

1. Lugg et al (1995) Endocrinology 136:1495-1501
2. Penson et al (1995) Am J Physiol, submitted
3. Penson et al (1995) Endocrinology, submitted
4. Garban et al (1995) Am J Physiol 268:H467-H475
5. Vernet et al (1995) Endocrinology, 136:in press
6. Garban et al (1995) in writing
7. Xie et al (1995) Am. Soc. Androl. Meet., submitted

For the study of long-term conditions related to impotence risk factors, very old Fischer 344 rats (30 month old), or spontaneously diabetic BB rats (Types I and II) with diabetes for over 5 months, wer used. Other groups included adult and old (20 month old) rats submitted to passive smoking for 2 months. Controls were untreated Fischer 344 adult and old rats, and age matched diabetic resistant BB rats.

In all cases the erectile function was measured by EFS as above, and, in the BB rats only, by

determining the erectile reflexes upon manipulation of the penis in the restrained non-anesthetized animal (number of cups and flips). The first detects cavernosal nerve compromise and the second peripheral neuropathies (dorsal nerve). With the exception of passive smoking, all other treatments or conditions impaired one or the other of the erectile parameters, showing a clear erectile dysfunction that can be used as a model for the corresponding situation in men (Fig. 25).

Penile NOS activity was measured in the cytosol by the L-arginine/citrulline conversion assay, as described in previous examples. In all cases without exception (Fig. 25), there was 50% or higher penile NOS reduction. Penile NOS content (the amount of NOS protein present, irrespective of its activity) was visualized by Western Blot and estimated by a semi-quantitative densitometric assay with a suitable conventional computer program. An antibody against the human nNOS isoform was used throughout because nNOS is the only isoform previously known to be naturally occurring in the penis.

In the case of short-term treatments, penile nNOS content (Table II) was unaffected, thus suggesting that an androgen-dependent inhibition of NOS activity is the main cause of the erectile dysfunction, coupled with other effects on the erectile mechanism. In the long-term conditions, involving neuropathies and fibrosis of the penile tissue, there is a clear reduction of penile nNOS. This includes one situation (smoking) where erection is not affected, probably because a compensatory mechanism based on non NO-ancillary pathways is activated during a long-term process.

The results obtained indicate clearly that a decrease of penile NOS is associated with erectile dysfunction in the rat, and that the therapeutic increase of NO synthesis by iNOS induction or iNOS gene therapy will ameliorate erectile dysfunction in the rat. These results also support our iNOS therapy procedures for the treatment of impotence in men.

INDUSTRIAL (MEDICAL/TREATMENT) APPLICABILITY.

It is clear that the products and methods of the invention will have wide ranging applicability to the treatment of vasculogenic erectile dysfunction in humans, not only in older patients, but those of all ages experiencing such dysfunction. The routes of administration of iNOS biological agents in accord with this invention depend on the class of product to be considered and are tabulated in Table III below. The main criteria are as follows:

A) Inducer mixes, of the type presented under Examples 3 and 4, cannot be applied systemically because of the risk of general induction of iNOS in organs other than the penis and the resulting systemic effects if iNOS is activated in each organ. The inducer mix is delivered directly into the corpora cavernosa in a continuous fashion for a short period (few days), because of the potential unstability of the cytokines. The effect will not be immediate, but last for a period ranging from a few days to weeks, and may require repeated administration.

B) Penile specific iNOS cDNA preparations, of the class presented under Examples 7 and 8, are based on: a) non-targeted, non-organ or tissue specific expression constructs, in liposome or other suitable pharmacologically acceptable formulations (indicated with -- on Table III); or b) organ or tissue specific expression constructs, in adenovirus or retrovirus formulations (indicated with TS on Table III). Both types of constructs are intended to provide a non-immediate medium-duration effect, depending on

the stable expression of an episomal or integrated foreign iNOS cDNA for days, weeks or months, mainly through intermittent applications.

The liposome formulations include a vector of the type presented under Examples 7 or 8, or other equivalent formulations with effective features and different lipid complexes, which may be expressed at low levels anywhere in the organism. Therefore, they are intended for direct penile application to avoid general effects. The adenovirus constructs provide a much more efficient transfer to even non-replicating cells, as those in the penile smooth muscle. These constructs also may be administered systemically, if a specific promoter assures preferential expression in the penis. For example, the constructs may be used with We propose the use of the smooth muscle ß-actin, the collagen, or alike, promoters, in order to target expression in the smooth muscle. The foreign iNOS protein will be restricted to fibrotic vascular tree (aging-related) and within this compartment it will only be functional in the penis, either because it requires activation by neurotransmitters released in the penis during sexual stimulation or because it represents a specific penile iNOS. Expression in other smooth muscle containing organs may also occur.

Also, replicating HPSMC in culture may be transformed by iNOS constructs in retroviral vectors, which may provide an even more efficient and stable integration, since retrovirus will induce the permanent modification of the HPSMC genome.

C) Penile smooth muscle cells (HPSMC) stably transfected with iNOS constructs, of the typ presented under Examples 6 and 7. They are transformed in vitro with either liposome, adenovirus, retrovirus, or similar, constructs, and then implanted in vivo locally in the corpora cavernosa, preferably at multiple locations to help insure uniform distribution and more effective production throughout the HPCC. No systemic administration is deemed feasible. This class of products provides a very targeted, highly organ-specific expression, with non-immediate sustained effects of the type described for class B (Ba and Bb in Table III). Cell cultures (HPSMC) obtained from the same patient may be preferred or even necessary, but stable cultures from a single source are expected to be functional according to the implant procedure.

D) Penile corpora cavernosa tissue transformed as for class C (Table III). They are particularly suited for direct implantation, a procedure where single cells are not adequate (see below). Likewise, the iNOS transfected corpora cavernosa pieces may be administered and work as for class C.

E) The recombinant iNOS protein made and purified as under Example 9, or the endogenous penile iNOS, may be applied, mainly locally. The intended use is for a short and immediate effect of the type obtained with locally administered vasodilators, as opposed to the other four classes. The protein is relatively unstable (limited shelf life), and requires repeated administration (non-stable transformation). Systemic routes would require that the protein is only active in the penis.

TABLE III

ROUTES OF ADMINISTRATION FOR iNOS BIOLOGICAL AGENTS FOR THE TREATMENT OF ERECTILE DYSFUNCTION

		cDNA					
		INDUCERS (A)	-- (Ba)	TS (Bb)	CELLS (C)	TISSUE (D)	PROTEIN (E)
	I. SYSTEMIC						
	1. Oral (Pill/tablet)			X			X
	2. Injection (Subcutaneous)			X			X
	II. LOCAL (P)						
	1. Injection (Direct)		X	X	X*		X
	2. Injection (Device)		X	X			
	3. Infusion (Pump)	X	X	X			
	4. Intraurethral (Pellet)	X	X	X			X
	5. Pellets and related (non-urethral)	X		X			
	6. Patch			X			X
	7. Rubbing (Ointment, cream, etc)			X			X
	8. Direct implant					X	
	9. Confined implant (Microcapsules, etc)				X	X	

* Optional.

As noted in the Table, the specific routes of administration can be classified as follows:

I) Systemic: restricted to targeted iNOS cDNA (adenovirus/tissue or organ specific promoter), and potentially to iNOS protein. It is self-administered by the patient, and may be either: 1) oral (preferential), or 2) subcutaneous or intravenous injection. In the case of the cDNA it is expected to may require

intermittent repetition, but not linked to the moment of sexual intercourse. In the case of the protein, administration would have to be immediately before sexual intercourse.

II) Local: depends on direct administration to the penis, and it is applicable to all classes of iNOS biological agents and to both types of timing for treatment in relation to sexual intercourse, as described above. Some of the prolonged effect treatment may require both physician and laboratory intervention.

1) Direct self-injection into the base of a temporarily constricted corpora cavernosa, as under Example 6, using the procedure applied for vasoactive compounds. This procedure is reserved for iNOS constructs, and may be for transfected HPSMC, although the latter may colonize locations other than the penis. It is also suitable for iNOS protein. The injection is done intermittently and at a timing independent from sexual intercourse, except for the iNOS protein.

2) Direct self-injection as for 1), but through a special device implanted by the physician, which allows repeated injections without the need of piercing with a needle at each time. Applications as for 1).

3) Infusion pump implanted by a physician in the penile or pubic region for continuous slow-release delivery directly into the corpora cavernosa. Adequate for inducers and cDNA, and will require a procedure for replenishment of iNOS agent.

4) Self-introduced intraurethral pellet, for an immediate (iNOS protein) or slow (inducers, iNOS constructs) delivery. However, the compounds remain less confined to the corpora cavernosa than in procedures 1-3.

5) Slow-delivery pellets or related, implanted in the corpora cavernosa by physician, which is suitable for inducers or targeted iNOS constructs, with the advantage of lower systemic diffusion.

6) Self-applied slow- or fast-delivery patches on penile or adjacent skin, for iNOS targeted constructs and protein. That is not considered advisable for inducers, and may be applied either pre-sexual intercourse (protein), or independent from it (constructs)

7) Self-applied slow- or fast-delivery rubbing preparation on penile glans, for targeted iNOS constructs or protein, in the form of ointment, lotion, cream, etc. This may be applied either pre-sexual intercourse (protein) or independent from it (constructs).

8) Direct implant of iNOS transformed corpora cavernosa smooth muscle tissue pieces from the same patient, done by physician. Tissue is transformed ex vivo in laboratory and re-implanted in patient. This is reserved for adenovirus transformed tissue (non-replicating), and is expected to provide the longest duration of effects. It has the advantage of being independent from sexual intercourse.

9) Confined implant of ex vivo transformed HPSMC from the same patient or from established cultures from other patients, done by physician and laboratory. Retroviral vectors are preferred for genomic integration and the procedure is independent from sexual intercourse. Single cells are maintained together in artificial compartments seeded in the corpora cavernosa, avoiding cell dispersion by the circulation. The carrier material may facilitate tissue colonization (degradable pellets), or create a physical semipermeable barrier between the transplanted cells and the host immune system (microcapsules of alginate or equivalents; dialysis membrane compartments; etc.). This is expected to provide the longest duration of effects.

The strategy devised above for iNOS-related biological agents for the treatment of erectile

dysfunction is readily applicable for nNOS and eNOS-related biological agents. The main differences are that no inducer treatment will be applicable because these are constitutive isozymes, and that the exogenous material will be subjected to the same physiological regulation affecting the endogenous nNOS, and possibly eNOS, in the penis. Thus, only local delivery systems are feasible, in order to avoid systemic effects. In addition, the use of biological activators of nNOS and eNOS may be warranted in those cases presented under Example 8, where nNOS (and possibly eNOS) content is not affected by the pathological condition. These modulators may include NOS co-factors (tetrahydrobiopterine, coenzymes, etc), substrate (L-arginine), dimerization inducers, and related materials, to be applied locally in the penis.

In summary, the preferred embodiment of our invention is based on the in vivo local continuous treatment of the penis with a mix of iNOS inducers by single or repeated local administration of constructs of iNOS, cDNA, or iNOS recombinant protein; or implants of genetically engineered penile cells or tissues hyperexpressing iNOS, to produce a considerable stimulation of the erectile response accompanied by an increase of penile NOS, which our evidence shows is the iNOS isozyme. The localized in vivo treatment surprisingly does not cause undesirable side effects that would be expected from systemic administration of iNOS inducers, and keeps the penile NOS actively under physiological control since no erection (priapism) is elicited in the absence of nerve stimulation. The demonstration and characterization of iNOS at the mRNA and protein levels in cells and tissue from the rat and human corpora cavernosa smooth muscle indicates that this unique isozyme plays a physiological role in the penis, and permits cloning its cDNA. Our evidence herein indicates penile cNOS is a distinct isozyme or species from other rat iNOS, including the vascular smooth muscle. Accordingly, our invention includes use of the corresponding recombinant iNOS cDNA, and those obtained from other species and penile cell types, which are effective in increasing NOS synthesis in the penis under physiological control. Our invention provides an improved method for raising penile NOS levels to treat erectile dysfunction case.

In addition, the scope of this invention may be extended to the treatment of erectile dysfunctions by administering locally to the penis: (a) constructs of nNOS or eNOS; (b)their respective CDNAs; (b) their respective proteins; (c) penile or tissue genetically engineered to express nNOS or eNOS; or (d) biological modulators of NOS activity, in such a way that they remain under psychological control and do not cause undesirable side effects. A systemic administration is also proposed for certain cases, preferably in association with tissue specific regulators.

It should be understood that various modifications within the scope of this invention can be made by one of ordinary skill in the art without departing from the spirit thereof. We therefore wish our invention to be defined by the scope of the appended claims as broadly as the prior art will permit, and in view of the specification if need be.

APPENDIX A

Complete nucleotide sequence of the inducible nitric oxide synthase from rat penile smooth muscle cells

```
   1  .......... .......... .......... .......... ...GAAACTT
  51  CTCAGCCACC TTGGTGAGGG GACTGGACTT TTAGAGACGC TTCTGAGGTT
 101  CCTCAGGCTT GGGTCTTGTT AGCCTAGTCA ACTACAAGCC CCACGGAGAA
 151  CAGCAGAGTT GGTGCAGAAG CACAAAGTCA CAGACATGGC TTGCCCCTGG
 201  AAGTTTCTCT TCAGAGTCAA ATCCTACCAA GGTGACCTGA AAGAGGAAAA
 251  GGACATTAAC AACAACGTGG AGAAAACCCC AGGTGCTATT CCCAGCCCAA
 301  CAACACAGGA TGACCCTAAG AGTCACAAGC ATCAAAATGG TTTCCCCCAG
 351  TTCCTCACTG GGACTGCACA GAATGTTCCA GAATCCCTGG ACAAGCTGCA
 401  TGTGACTCCA TCGACCCGCC CACAGCACGT GAGGATCAAA AACTGGGGCA
 451  ATGGAGAGAT TTTTCACGAC ACCCTTCACC ACAAGGCCAC CTCGGATATC
 501  TCTTGCAAGT CCAAATTATG CATGGGGTCC ATCATGAACT CCAAGAGTTT
 551  GACCAGAGGA CCCAGAGACA AGCCCACCCC AGTGGAGGAG CTTCTGCCTC
 601  AAGCCATTGA ATTCATTAAC CAGTATTATG GCTCCTTCAA AGAGGCAAAA
 651  ATAGAGGAAC ATCTGGCCAG GCTGGAAGCC GTAACAAAGG AAATAGAAAC
 701  AACAGGAACC TACCAGCTCA CTCTGGATGA GCTCATCTTT GCCACCAAGA
 751  TGGCCTGGAG GAACGCCCCT CGCTGCATCG GCAGGATTCA GTGGTCCAAC
 801  CTGCAGGTCT TCGATGCCCG GAGCTGTAGC ACTGCATCAG AAATGTTCCA
 851  GCATATCTGC AGACACATAC TTTACGCCAC TAACAGTGGC AACATCAGGT
 901  CGGCCATTAC TGTGTTCCCC CAGCGGAGCG ATGGGAAGCA TGACTTCCGG
```

APPENDIX A (cont.)

951 ATCTGGAATT CCCAGCTCAT CCGGTACGCT GGCTACCAGA TGACCGATGG

1001 CACCATCAGA GGGGATCCTG CCACCTTGGA GTTCACCCAG TTGTGCATCG

1051 ACCTGGGCTG GAAGCCCCGC TATGGCCGCT TCGATGTGCT GCCTCTGGTC

1101 CTGCAGGCTC ACGGTCAAGA TCCAGAGGTC TTTGAAATCC CTCCTGATCT

1151 TGTGCTGGAG GTGACCATGG AGCATCCCAA GTACGAGTGG TTCCAGGAGC

1201 TCGGGCTGAA GTGGTATGCG CTGCCTGCCG TGGCCAACAT GCTCCTGGAG

1251 GTGGGTGGCC TCGAGTTCCC AGCCTGCCCC TTCAATGGTT GGTACATGGG

1301 CACCGAGATT GGAGTCCGAG ACTTCTGTGA CACACAGCGC TACAACATCC

1351 TGGAGGAAGT GGGCAGGAGG ATGGGCCTGG AGACCCACAC ACTGGCCTCC

1401 CTCTGGAAAG ACCGGGCTGT CACCGAGATC AATGCAGCTG TGCTCCATAG

1451 TTTTCAGAAG CAGAATGTGA CCATCATGGA CCACCACACA GCCTCAGAGT

1501 CCTTCATGAA GCACATGCAG AATGAGTACC GGGCCCGAGG AGGCTGCCCT

1551 GCAGACTGGA TTTGGCTGGT CCCTCCGGTG TCCGGGAGCA TCACCCCTGT

1601 GTTCCACCAG GAGATGTTGA ACTACGTCCT ATCTCCATTC TACTACTACC

1651 AGATCGAGCC CTGGAAGACC CACATCTGGC AGGATGAGAA GCTGAGGCCC

1701 AGGAGGAGAG AGATCCGGTT CACAGTCTTG GTGAAAGCGG TGTTCTTTGC

1751 TTCTGTGCTA ATGCGGAAGG TCATGGCTTC CCGCGTCAGA GCCACAGTCC

1801 TCTTTGCTAC TGAGACAGGA AAGTCGGAAG CGCTAGCCAG GGACCTGGCT

1851 GCCTTGTTCA GCTACGCCTT CAACACCAAG GTTGTCTGCA TGGAACAGTA

1901 TAAGGCAAAC ACCTTGGAAG AGGAACAACT ACTGCTGGTG GTGACAAGCA

1951 CATTTGGCAA TGGAGACTGC CCCAGCAATG GGCAGACTCT GAAGAAATCT

2001 CTGTTCATGA TGAAAGAACT CGGGCATACC TTCAGGTATG CGGTATTTGG

2051 CCTGGGCTCC AGCATGTACC CTCAGTTCTG TGCCTTTGCT CATGACATCG

2101 ACCAGAAACT GTCTCACCTG GGAGCCTCCC AGCTTGCCCC AACCGGAGAA

2151 GGGGACGAAC TCAGCGGGCA GGAGGACGCC TTCCGCAGCT GGGCTGTGCA

2201 AACCTTCCGG GCAGCCTGTG AGACGTTCGA TGTTCGAAGC AAACATTGCA

2251 TTCAGATCCC GAAACGCTAC ACTTCCAACG CAACATGGGA GCCAGAGCAG

APPENDIX A (cont.)

2301 TACAAGCTCA CCCAGAGCCC AGAGTCTCTA GACCTCAACA AAGCTCTCAG

2351 GAGCATCCAC GCCAAGAACG TGTTCACCAT GAGGCTGAAA TCCCTCCAGA

2401 ATCTGCAGAG TGAGAAGTCC AGCCGCACCA CCCTCCTTGT TCAACTCACC

2451 TTCGAGGGCA GCCGAGGCCC CAGCTACCTA CCTGGGGAAC ACCTGGGGAT

2501 TTTCCCAGGC AACCAGACGG CCCTGGTGCA AGGGATCTTG GAGCGAGTTG

2551 TGGATTGTTC TTCGCCAGAC CAAACTGTGT GCCTGGAGGT TCTAGATGAG

2601 AGTGGCAGCT ACTGGGTCAA AGACAAGAGG CTTCCCCCCT GCTCACTCAG

2651 GCAAGCCCTC ACCTACTTCC TGGACATCAC TACCCCTCCC ACCCAGCTGC

2701 AGCTCCACAA GCTGGCCCGC TTTGCCACGG AAGAGACGCA CAGGCAGAGG

2751 TTGGAGGCCT TGTGTCAGCC CTCAGAGTAC AACGATTGGA AGTTCAGCAA

2801 CAACCCCACG TTCCTGGAGG TGCTGGAAGA GTTCCCATCA TTGCGTGTGC

2851 CTGCTGCCTT CCTGCTGTCG CAGCTCCCCA TTCTGAAGCC CCGCTACTAC

2901 TCCATCAGCT CCTCCCAGGA CCACACCCCC TCGGAGGTCC ACCTCACTGT

2951 GGCTGTGGTC ACCTATCGCA CCCGAGATGG TCAGGGTCCC CTGCACCATG

3001 GCGTCTGCAG CACTTGGATC AATAACCTGA AGCCCGAAGA CCCAGTGCCC

3051 TGCTTTGTGC GGAGTGTCAG TGGCTTCCAG CTCCCTGAGG ACCCCTCCCA

3101 GCCCTGCATC CTCATTGGGC CCGGTACAGG CATTGCCCCC TTCCGAAGTT

3151 TCTGGCAGCA GCGGCTCCAT GACTCTCAGC ACAGAGGGCT CAAAGGAGGC

3201 CGCATGACCT TGGTGTTTGG GTGCAGGCAC CCAGAGGAGG ACCACCTCTA

3251 TCAGGAAGAA ATGCAGGAGA TGGTCCGCAA GGGAGTGTTG TTCCAGGTGC

3301 ACACAGGCTA CTCCCGGCTG CCCGGAAAAC CCAAGGTCTA CGTTCAAGAC

3351 ATCCTGCAGA AAGAGCTGGC CGACGAGGTG TTCAGCGTGC TCCACGGGGA

3401 GCAGGGCCAC CTCTATGTTT GTGGCGATGT GCGCATGGCT CGGGATGTGG

3451 CTACCACTTT GAAGAAGCTG GTGGCCGCCA AGCTGAACTT GAGTGAGGAG

3501 CAGGTTGAGG ATTACTTCTT CCAGCTCAAG AGCCAGAAAC GTTATCATGA

3551 GGATATCTTC GGTGCGGTCT TTTCCTATGG AGCAAAAAAG GGCAACACCT

APPENDIX A (cont.)

3601 TGGAGGAGCC CAAAGGCACA AGACTCTGAC ACCCAGAAGA GTTACAGCAT

3651 CTGGCCCTAA ATAAAATGAC AGTGAGGGTT TGGAGAGACA GAAGTGCGAT

3701 CCCCCCCAAC CCCCTCACAT CATCTCCCCT CCTCTACCCT ACCAAGTAGT

3751 ATTGTTCTAT TGTGGACTAC TGAATCTCTC TCCTCTCCCC CGTCCCCTAT

3801 TCTCCCTTCC CTCCCGTTTT CTCTGCCCTC CCCCACATTC TCTTTCCTTT

3851 GCCTCATACT TCCTCAGAGC TGAGAGCAGA GGAAAATGAA CCACCCGACT

3901 GAAGCACTTT GGGTGACCAC CAGGAGGCGC CATCCCGCTG CTCTAATATT

3951 TAGCTGCATT GTGTACAGAT ATTTATACTT TGTATTTAAG AAAACATACC

4001 TTCGTCCACT CCCAACGACC GCTTGGCCCT TCCGTGTATA ATTCCTTGAT

4051 GAAGATATTT ATATAAAATG CATTTTATTT TAATAAAATG AGTGTTTGAT

4101 CA..... 4119

APPENDIX B

Complete aminoacid sequence of the inducible NOS from rat penile smooth muscle cells (RPSMC iNOS)

```
  1  MACPWKFLFR VKSYQGDLKE EKDINNNVEK TPGAIPSPTT QDDPKSHKHQ
 51  NGFPQFLTGT AQNVPESLDK LHVTPSTRPQ HVRIKNWGNG EIFHDTLHHK
101  ATSDISCKSK LCMGSIMNSK SLTRGPRDKP TPVEELLPQA IEFINQYYGS
151  FKEAKIEEHL ARLEAVTKEI ETTGTYQLTL DELIFATKMA WRNAPRCIGR
201  IQWSNLQVFD ARSCSTASEM FQHICRHILY ATNSGNIRSA ITVFPQRSDG
251  KHDFRIWNSQ LIRYAGYQMS DGTIRGDPAT LEFTQLCIDL GWKPRYGRFD
                        P
301  VLPLVLQAHG QDPEVFEIPP DLVLEVTMEH PKYEWFQELG LKWYALPAVA
                                                        A
351  NMLLEVGGLE FPACPFNGWY MGTEIGVRDF CDTQRYNILE EVGRRMGLET
401  HTLASLWKDR AVTEINAAVL HSFQKQNVTI MDHHTASESF MKHMQNEYRA
451  RGGCPADWIW LVPPVSGSIT PVFHQEMLNY VLSPFYYYQI EPWKTHIWQD
501  EKLRPRRREI RFTVLVKAVF FASVLMRKVM ASRVRATVLF ATETGKSEAL
551  ARDLAALFSY AFNTKVVCME QYKANTLEEE QLLLVVTSTF GNGDCPSNGQ
                                                    V
601  TLKKSLFMMK ELGHTFRYAV FGLGSSMYPQ FCAFAHDIDQ KLSHLGASQL
```

APPENDIX B (cont.)

651 APTGEGDELS GQEDAFRSWA VQTFRAACET FDVRSKHCIQ IPKRYTSNAT

701 WEPEQYKLTQ SPESLDLNKA LRSIHAKNVF TMRLKSLQNL QSEKSSRTTL
P P

751 LVQLTFEGSR GPSYLPGEHL GIFPGNQTAL VQGILERVVD CSSPDQTVCL

801 EVLDESGSYW VKDKRLPPCS LRQALTYFLD ITTPPTQLQL HKLARFATEE

851 THRQRLEALC QPSEYNDWKF SNNPTFLEVL EEFPSLRVPA AFLLSQLPIL

901 KPRYYSISSS QDHTPSEVHL TVAVVTYRTR DGQGPLHHGV CSTWINNLKP

951 EDPVPCFVRS VSGFQLPEDP SQPCILIGPG TGIAPFRSFW QQRLHDSQHR

1001 GLKGGRMTLV FGCRHPEEDH LYQEEMQEMV RKGVLFQVHT GYSRLPGKPK

1051 VYVQDILQKE LADEVFSVLH GEQGHLYVCG DVRIARDVAT TLKKLVAAKL
M

1101 NLSEEQVEDY FFQLKSQKRY HEDIFGAVFS YGAKKGNTLE EPKGTRL*

APPENDIX C

Human iNOS Sequence List

APPENDIX D

Human iNOS cDNA Sequence List

CLAIMS

WE CLAIM:

1. A method of treatment of erectile dysfunction in a patient comprising the steps of:
   a) providing an agent which produces an increase in in vivo tissue iNOS level;
   b) introducing an effective amount of said iNOS agent into penis tissue.

2. An erectile dysfunction treatment method as in claim 1 wherein:
   a) said agent is selected from the group consisting essentially of iNOS inducers, iNOS protein, iNOS cDNA, and iNOS cDNA-transformed penile cells or iNOS cDNA-transformed tissue.

3. An erectile dysfunction treatment method as in claim 2 wherein said iNOS inducer is a penile iNOS inducer, said iNOS protein is a penile iNOS protein, said iNOS cDNA is penile iNOS cDNA, and said iNOS cDNA-transformed cells or tissue are penile cells or tissue.

4. An erectile dysfunction treatment method as in claim 1 wherein:
   a) said step of introduction includes introducing iNOS inducers directly into in vivo penis tissue in an intermittent, continuous or time release basis.

5. An erectile dysfunction treatment method as in claim 2 wherein:
   a) said inducers are selected from the group consisting essentially of bacterially lipopolysaccharide, interferon - γ, tumor necrosis factor - α, interleukin - 1β, and mixtures thereof.

6. An erectile dysfunction treatment method as in claim 2 wherein:
   a) said agent is iNOS protein produced in vitro.

7. An erectile dysfunction treatment method as in claim 6 wherein said iNOS protein is penile iNOS protein.

8. An erectile dysfunction treatment method as in claim 6 wherein said iNOS protein is administered locally by continuous infusion or repeated injection, topical application, intraurethral administration, microcapsules, or related procedures.

9. An erectile dysfunction treatment method as in claim 3 wherein:
   a) said agent is penile or other iNOS cDNA administered locally by continuous infusion or repeated injection, topical application, intraurethral administration, microcapsules, or related procedures, as well as by systemic routes.

10. An erectile dysfunction treatment method as in claim 3 wherein:

a) said agent is iNOS cDNA-transformed penile corpora cavernosa cells or tissue implanted directly into the corpora cavernosa or tissue implanted directly into the corpora cavernosa or retained in microcapsules, pellets or other procedures intended to limit the release of cells into the general circulation.

11. An erectile dysfunction treatment method as in claim 2 wherein:

a) said agent is penile or other iNOS or eNOS cDNAs administered locally by continuous infusion or repeated injection, topical application, intraurethral administration, microcapsules, or related procedures.

12. An erectile dysfunction treatment method as in claim 2 wherein:

a) said agent is iNOS or eNOS cDNA transferred penile corpora cavernosa cells or tissues implanted directly into the corpora cavernosa or tissue implanted directly into the corpora cavernosa or retained in microcapsules, pellets or other procedures intended to limit the release of cells into the general circulation.

13. An erectile dysfunction treatment method as in claim 2 wherein:

a) said agent is a biological modulator of endogenous or exogenous penile NOS.

14. A cDNA expression vector comprising plasmid pBS RPiNOS.

15. A DNA fragment consisting essentially of a structural gene encoding a protein selected from the group consisting of cNOS, nNOS, eNOS and iNOS.

16. A DNA fragment as in claim 15 wherein said DNA fragment is cDNA.

17. A DNA fragment as in claim 16 wherein the DNA fragment is recombinant DNA.

18. A DNA fragment as in claim 17 operably linked to a promoter to express said gene.

19. A recombinant host expressing a gene encoding a nitric oxide synthase as in claim 18.

20. A DNA sequence coding for induced nitric oxide synthase enzyme.

21. The sequence of claim 20 wherein the DNA is a cDNA.

22. The sequence of claim 20 derived from a natural source.

23. The sequenc of claim 22 derived from the group consisting of rat PSMC and human PSMC, said cDNA from rat PSMC having the DNA sequence, in a 5' to 3' direction, as shown in Appendix A, said cDNA from human PSMC having the DNA sequence in a 5' to 3' direction, as shown in Appendix D. [See Appendix A for Chemical Structure-Diagram.]

24. A cDNA expression vector which comprises:

a) a promoter fragment which functions in a host organism;

b) a cDNA segment, said cDNA codes for nitric oxide synthase enzyme;

c) said cDNA segment being in an orientation with the promoter fragment such that it is expressed in the host to produce a non-native induced nitric oxide synthase enzyme.

25. The expression vector of claim 24 wherein said host organisms are fetal cells, e. coliform, yeast or PSMC.

26. A transfected host organism transformed by the expression vector of claim 24.

27. A transfected host organism comprising:

a) a cell having disposed therein a DNA expression vector for coding nitric oxide synthase enzyme.

b) said cell is a microbial or mammalian cell.

28. An improved tissue, the tissue relying on the production of nitric oxide for normal function, the improvement comprising introduction of transfected cells to the host tissue, said cells having disposed therein a DNA expression vector coding for nitric oxide synthase.

29. Nitric oxide synthase enzymes derived from PSMC cDNA, said nitric oxide synthase enzyme selected from the group consisting of rat iNOS and human iNOS, said rat iNOS having the amino acid sequence as shown in Appendix B, and said human iNOS having the amino acid sequence as shown in Appendix C.

30. A eukaryotic expression vector:

(a) pc DNA3 RPiNOS consisting essentially of pcDNA3 containing a penile coding region, a plasmid and a promoter fraction.

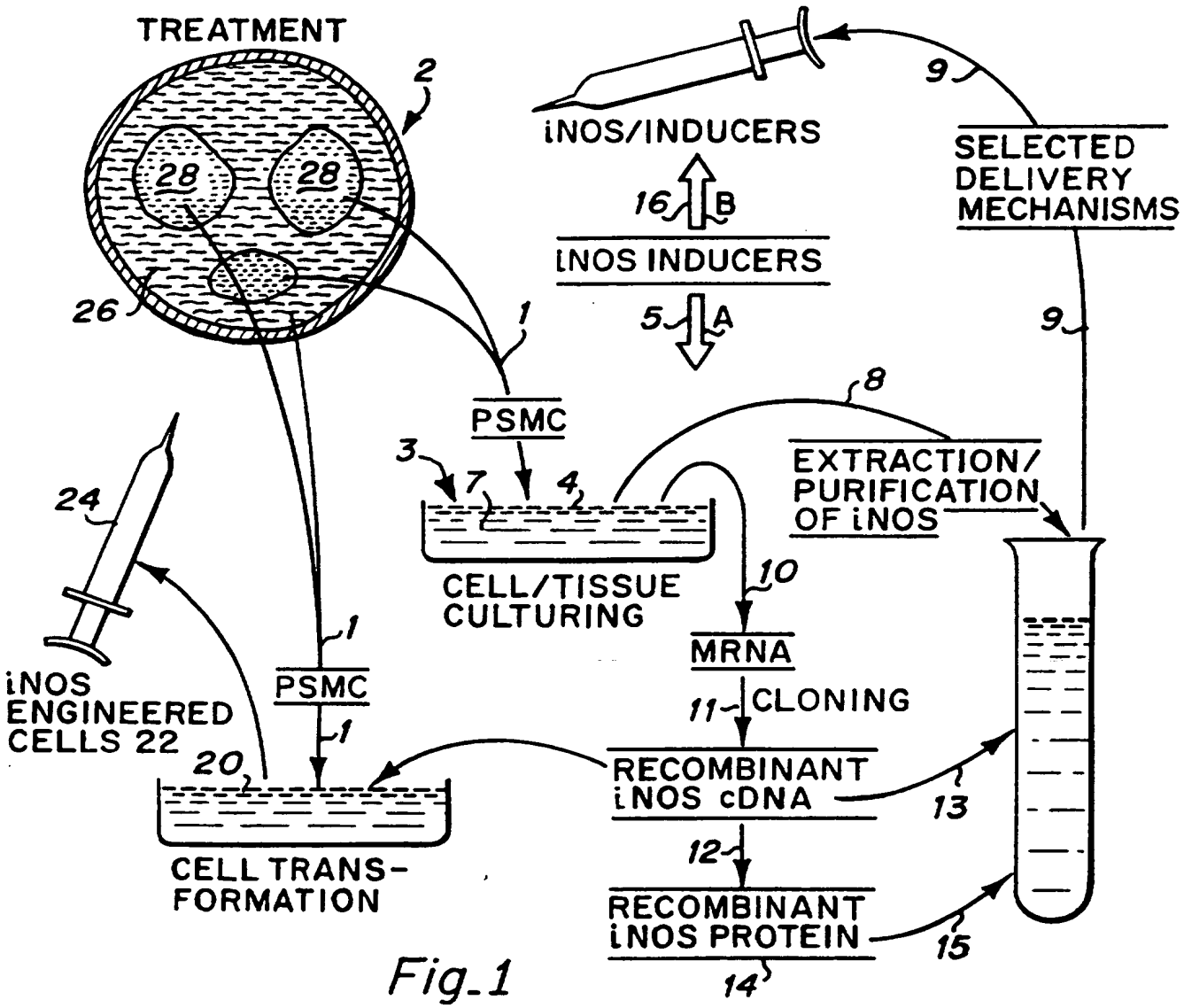
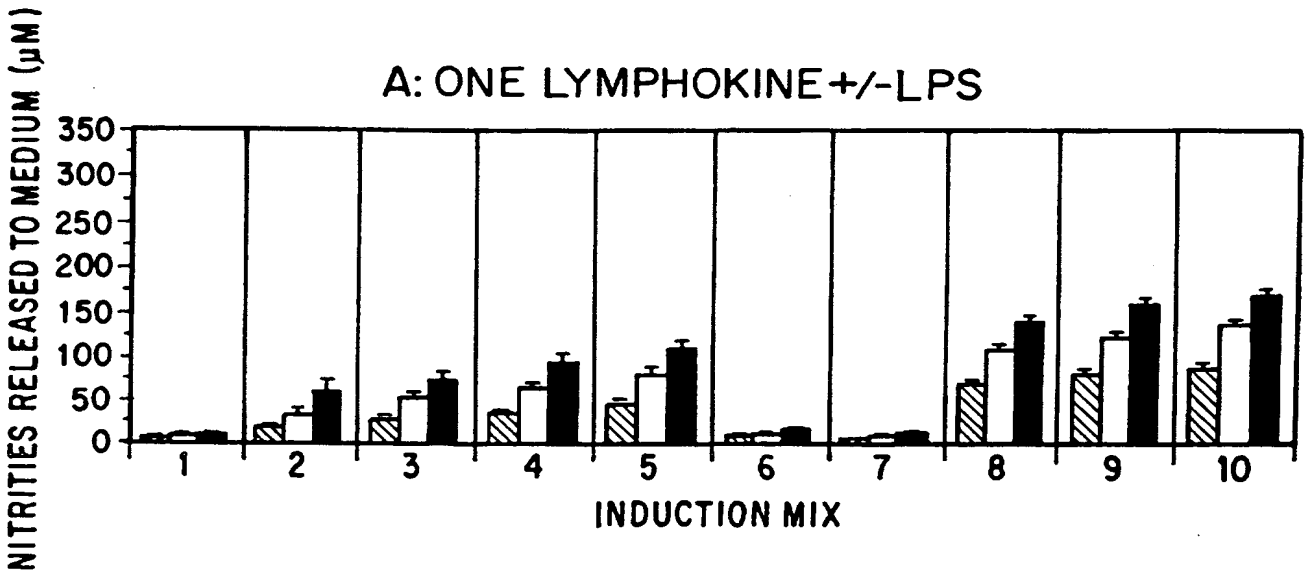
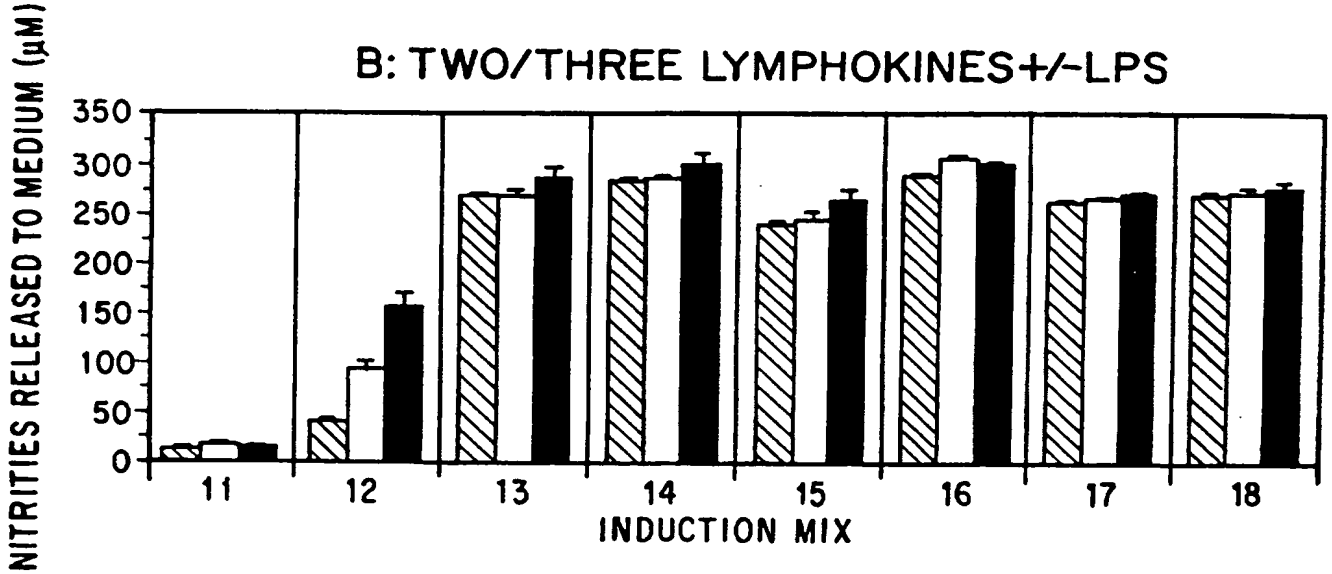

TREATMENT
2
28
28
26
1
PSMC
3
7
4
CELL/TISSUE CULTURING
10
MRNA
11
CLONING
RECOMBINANT iNOS cDNA
13
12
RECOMBINANT iNOS PROTEIN
14
15
iNOS/INDUCERS
16
B
iNOS INDUCERS
5
A
9
SELECTED DELIVERY MECHANISMS
8
EXTRACTION/ PURIFICATION OF iNOS
24
iNOS ENGINEERED CELLS 22
PSMC
1
20
CELL TRANS-FORMATION

Fig_1



□ = NO LPS; ▨ = 1UG/MI LPS; ■ = 10 UG/MI LPS.
OTHER ADDITIONS ARE LISTED IN THE CONCENTRATIONS INDICATED IN THE FOLLOWING ORDER INF-GAMMA (U/MI)/TNF-ALPHA (U/MI)/IL-BETA 1 (NG/MI) FOR EACH PANEL = 1:0/0/0; 2:50/0/0; 3:100/0/0; 4:250/0/0; 5:500/0/0; 6:0/200/0; 7:0/500/0; 8:0/0/5; 9:0/0/20; 10:0/0/100.

Fig_2



□=NO LPS; ▨=1 UG/MI LPS; ■=10 UG/MI LPS.
OTHER ADDITIONS ARE LISTED IN THE CONCENTRATIONS INDICATED IN THE FOLLOWING ORDER INF-GAMMA (U/MI) TNF-ALPHA (U/MI)/IL-BETA 1 (UG/MI) FOR EACH PANEL = 11: 0/0/0; 12: 250/0/0; 13: 250/500/20; 14: 250/3000/20; 15: 500/0/100; 16: 500/500/100; 17: 100/500/5; 18: 250/500/5

Fig_3

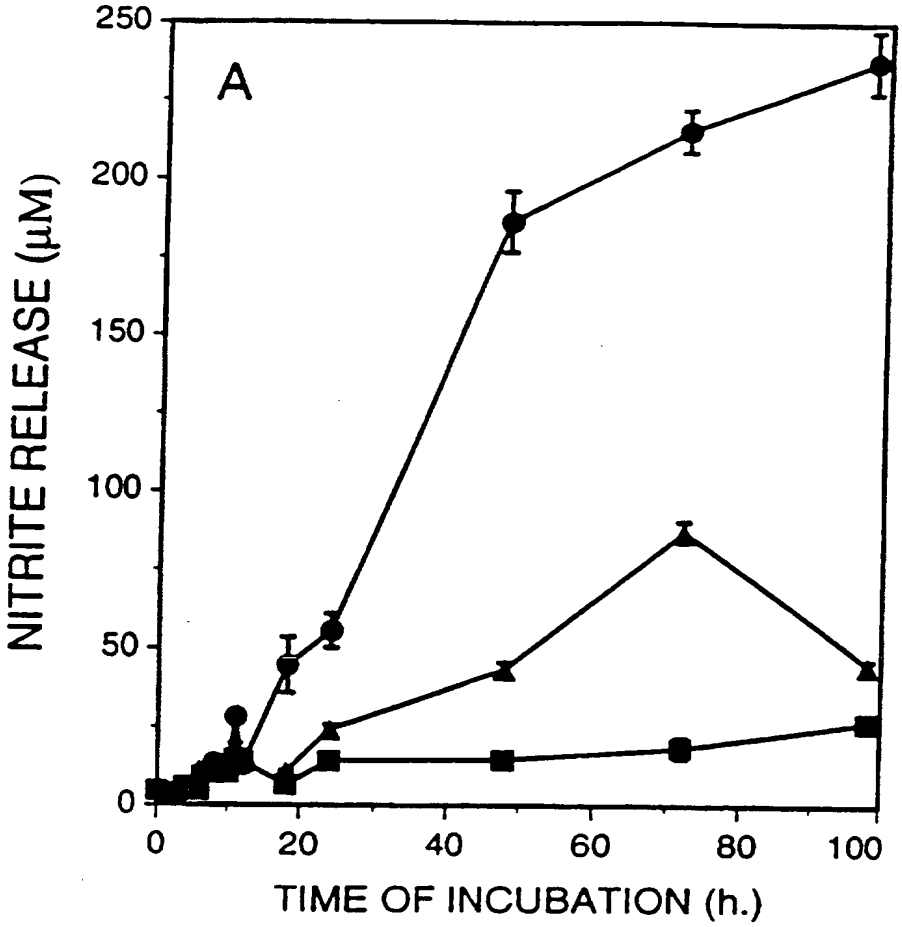

A
NITRITE RELEASE (µM)
250
200
150
100
50
0
0
20
40
60
80
100
TIME OF INCUBATION (h.)
control (non-induced)
control (induced)
L-NAME (induced)

Fig_4

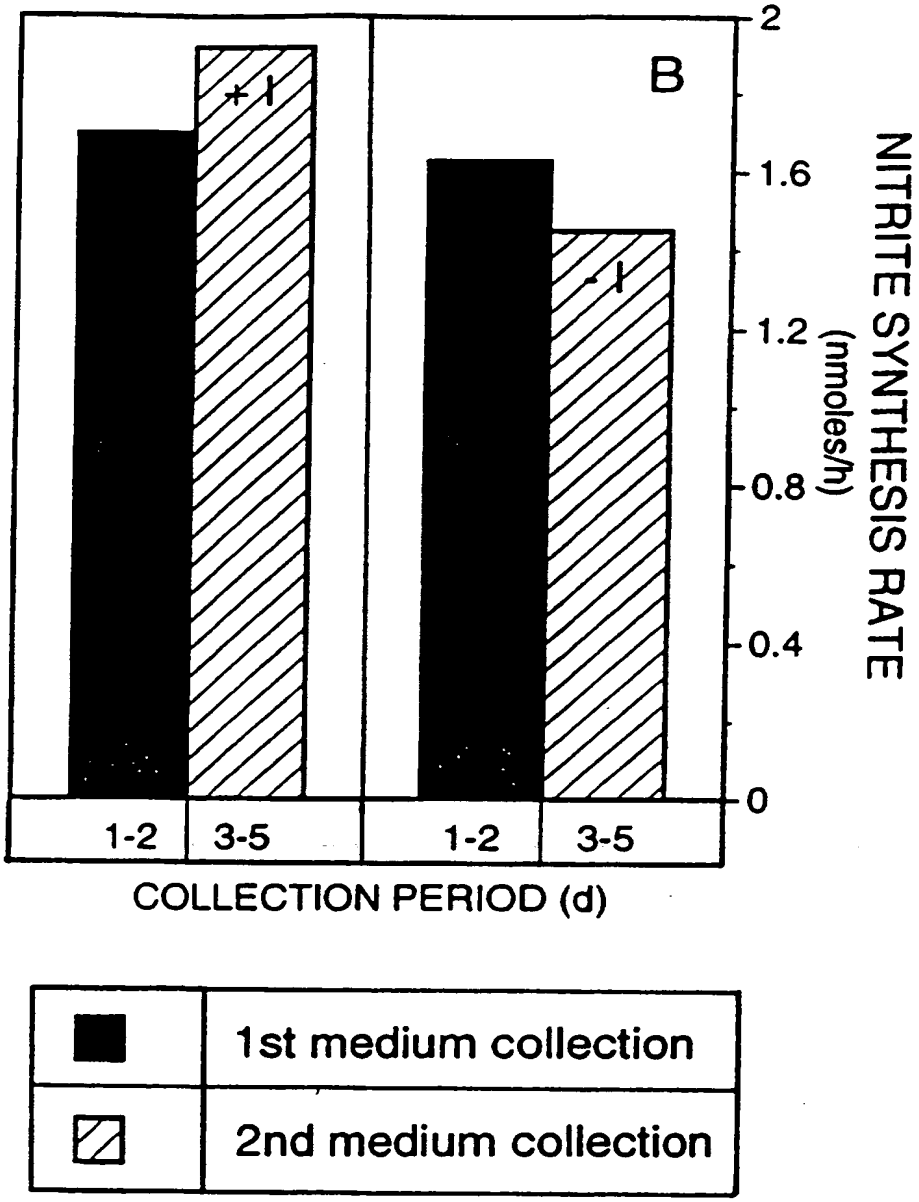

B
+I
-I
2
1.6
1.2
0.8
0.4
0
NITRITE SYNTHESIS RATE
(nmoles/h)
1-2
3-5
1-2
3-5
COLLECTION PERIOD (d)
1st medium collection
2nd medium collection

Fig.5

A

NORTHERN

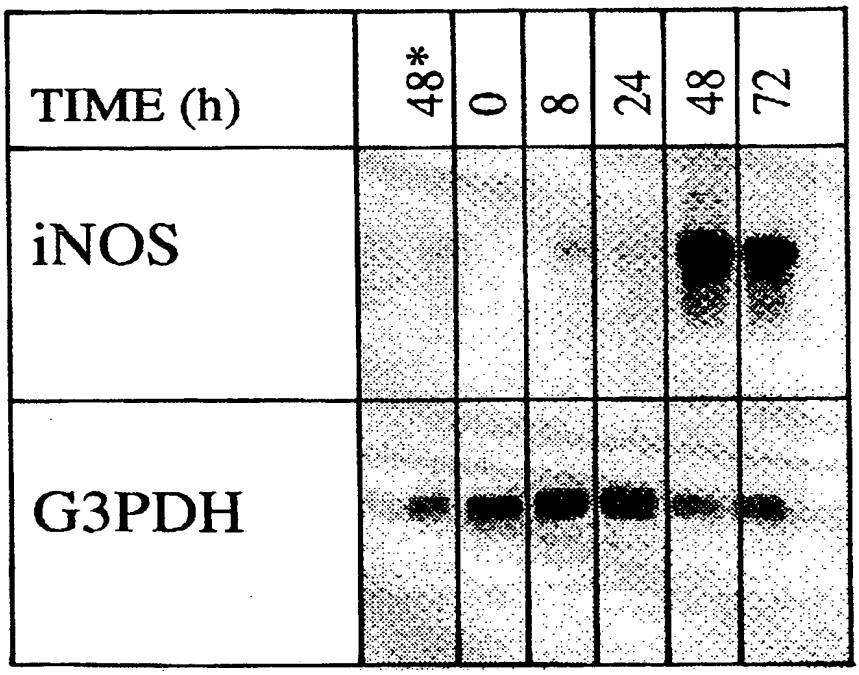


Fig. 6

B

WESTERN

LANE #	1	2	3
iNOS			

Fig. 7

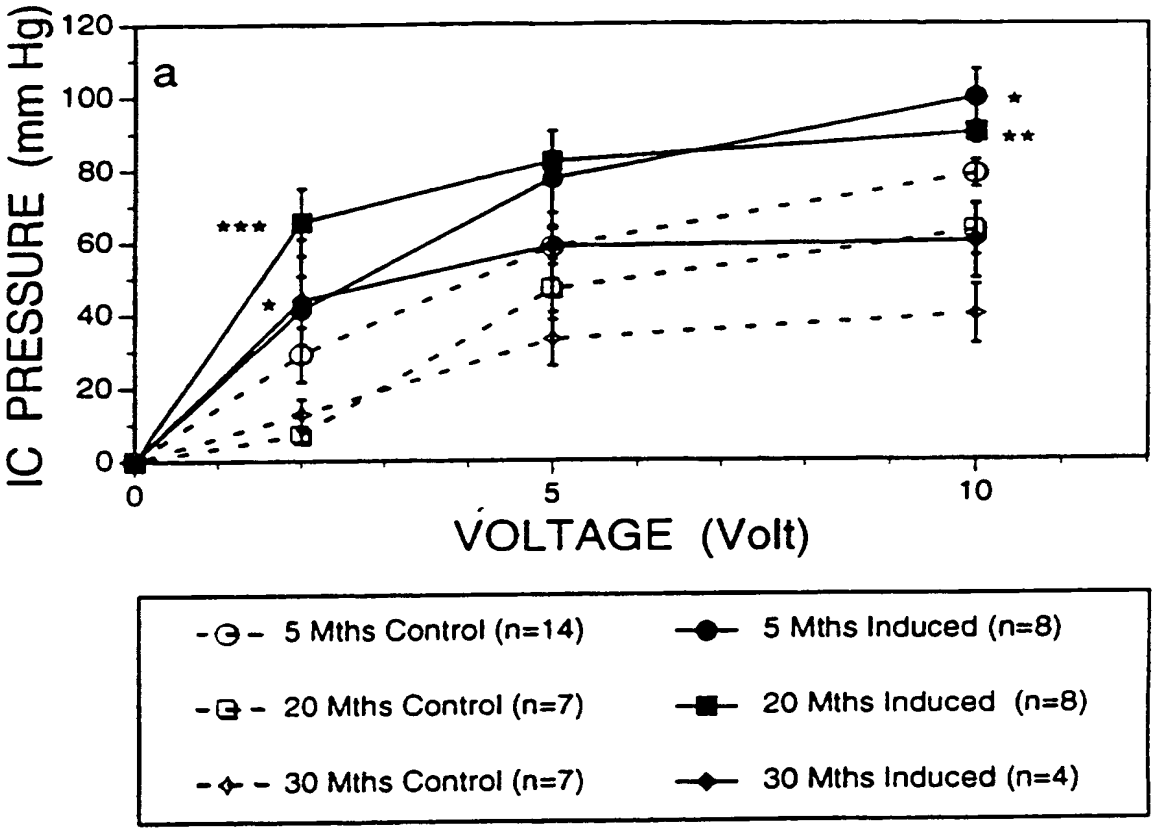

IC PRESSURE (mm Hg)
120
100
80
60
40
20
0
a
***
*
*
**
0
5
10
VOLTAGE (Volt)
5 Mths Control (n=14)
5 Mths Induced (n=8)
20 Mths Control (n=7)
20 Mths Induced (n=8)
30 Mths Control (n=7)
30 Mths Induced (n=4)

Fig_8

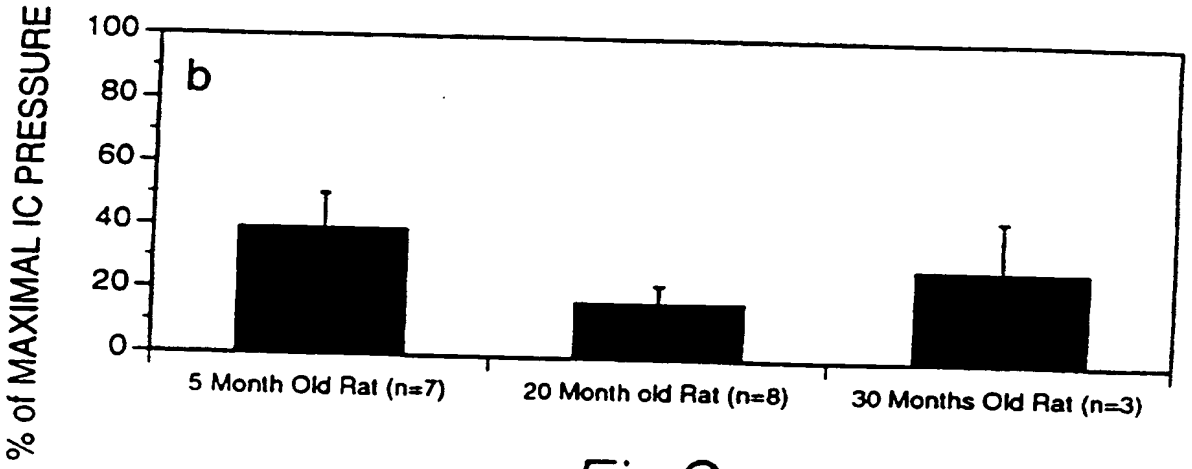

% of MAXIMAL IC PRESSURE
100
80
60
40
20
0
b
5 Month Old Rat (n=7)
20 Month old Rat (n=8)
30 Months Old Rat (n=3)

Fig_9

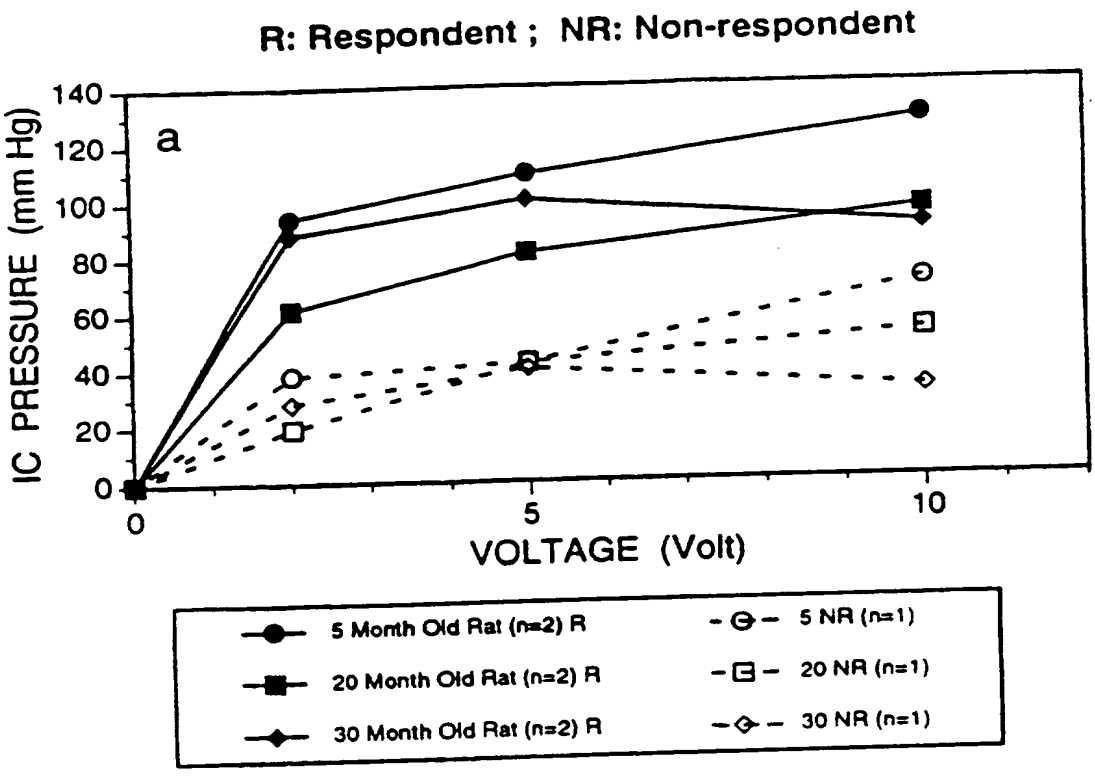

R: Respondent ; NR: Non-respondent
a
IC PRESSURE (mm Hg)
140
120
100
80
60
40
20
0
0
5
10
VOLTAGE (Volt)
5 Month Old Rat (n=2) R
20 Month Old Rat (n=2) R
30 Month Old Rat (n=2) R
5 NR (n=1)
20 NR (n=1)
30 NR (n=1)

Fig_10

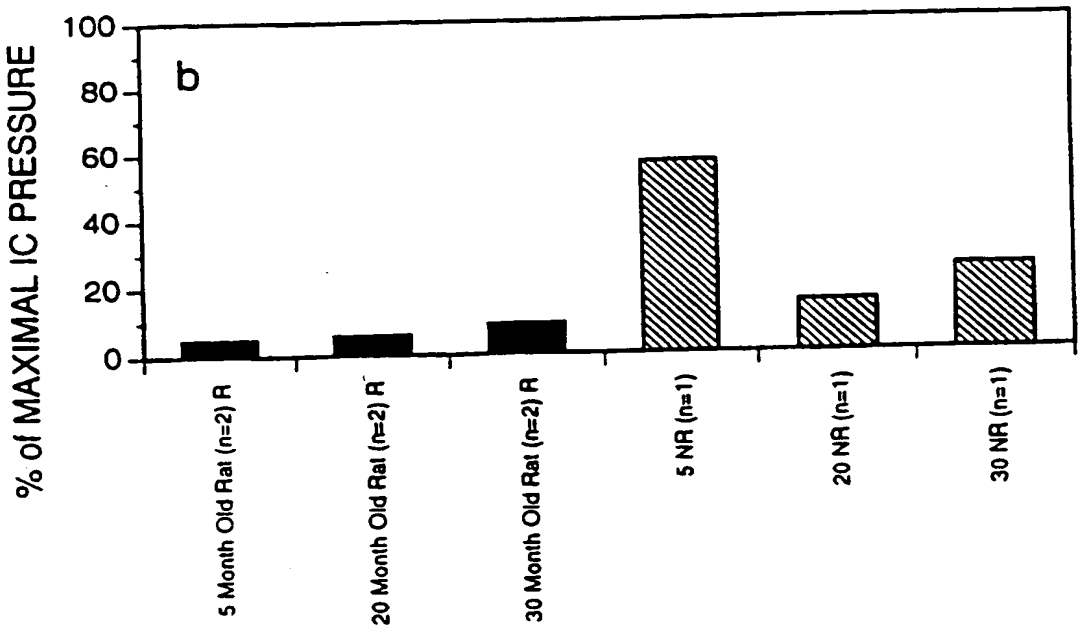

b
% of MAXIMAL IC PRESSURE
100
80
60
40
20
0
5 Month Old Rat (n=2) R
20 Month Old Rat (n=2) R
30 Month Old Rat (n=2) R
5 NR (n=1)
20 NR (n=1)
30 NR (n=1)

Fig_11

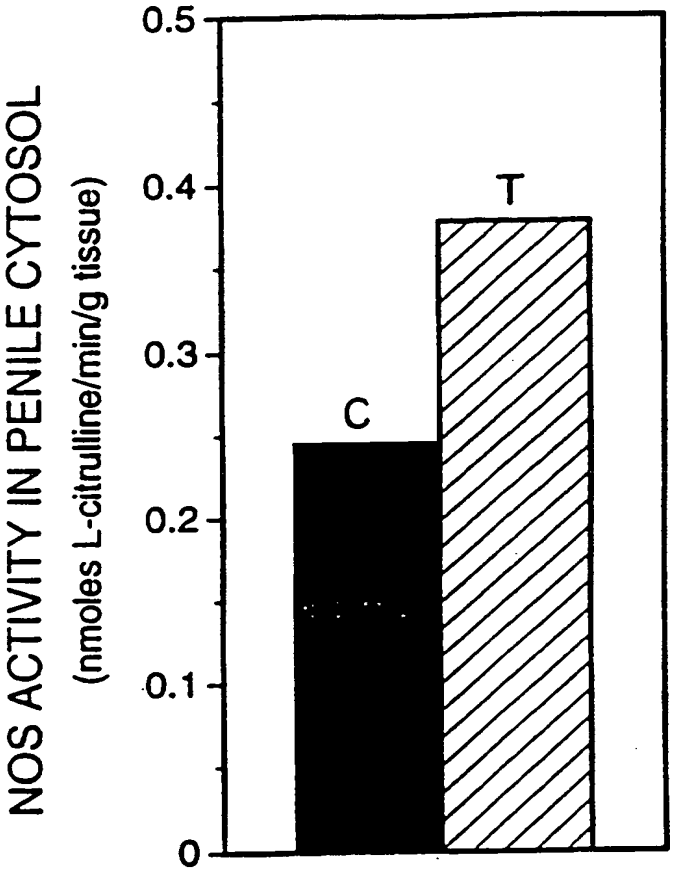

NOS ACTIVITY IN PENILE CYTOSOL
(nmoles L-citrulline/min/g tissue)
0.5
0.4
0.3
0.2
0.1
0
C
T

Fig_12

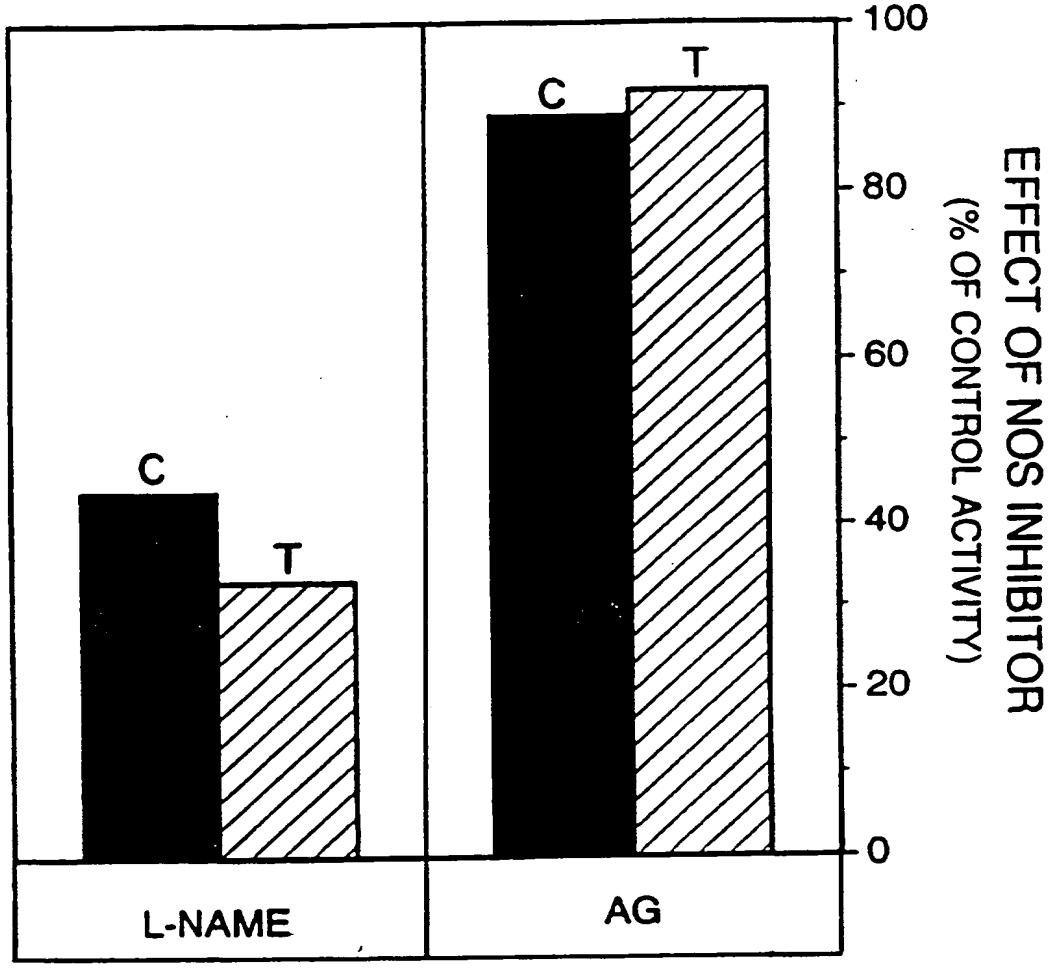

100
80
60
40
20
0
EFFECT OF NOS INHIBITOR
(% OF CONTROL ACTIVITY)
C
T
C
T
L-NAME
AG

Fig_13

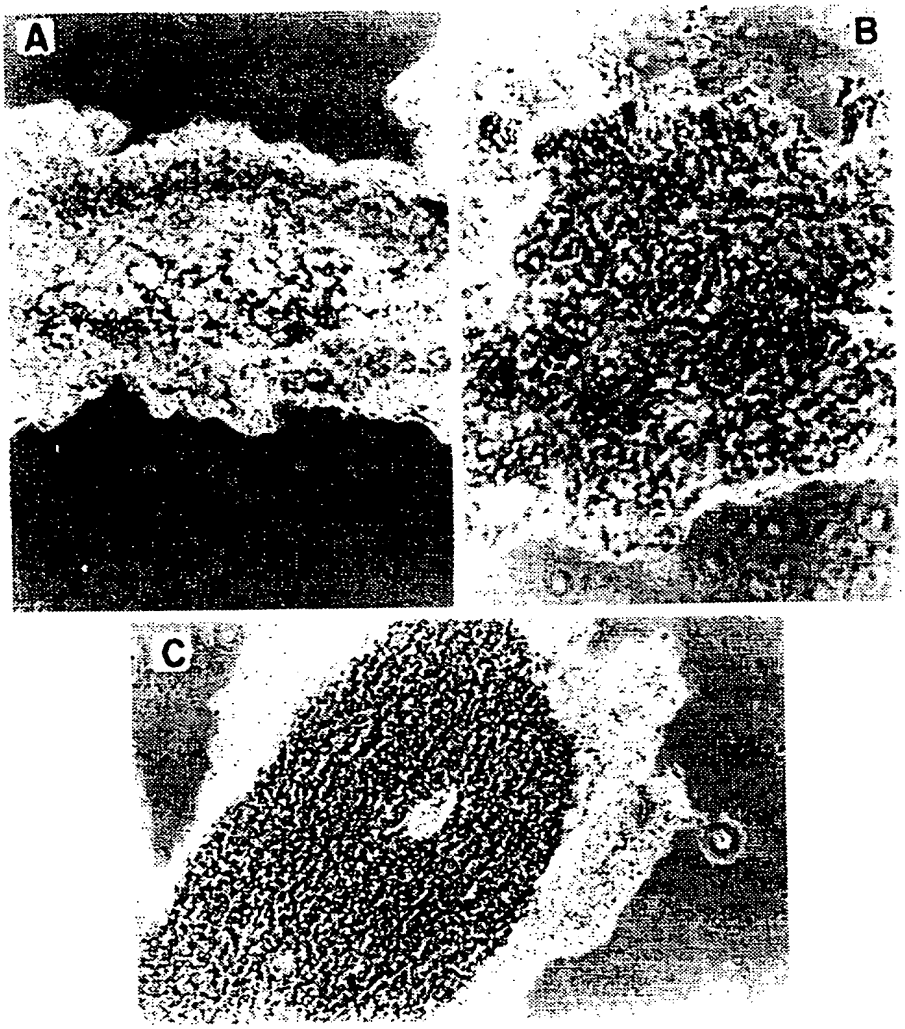


Fig_14

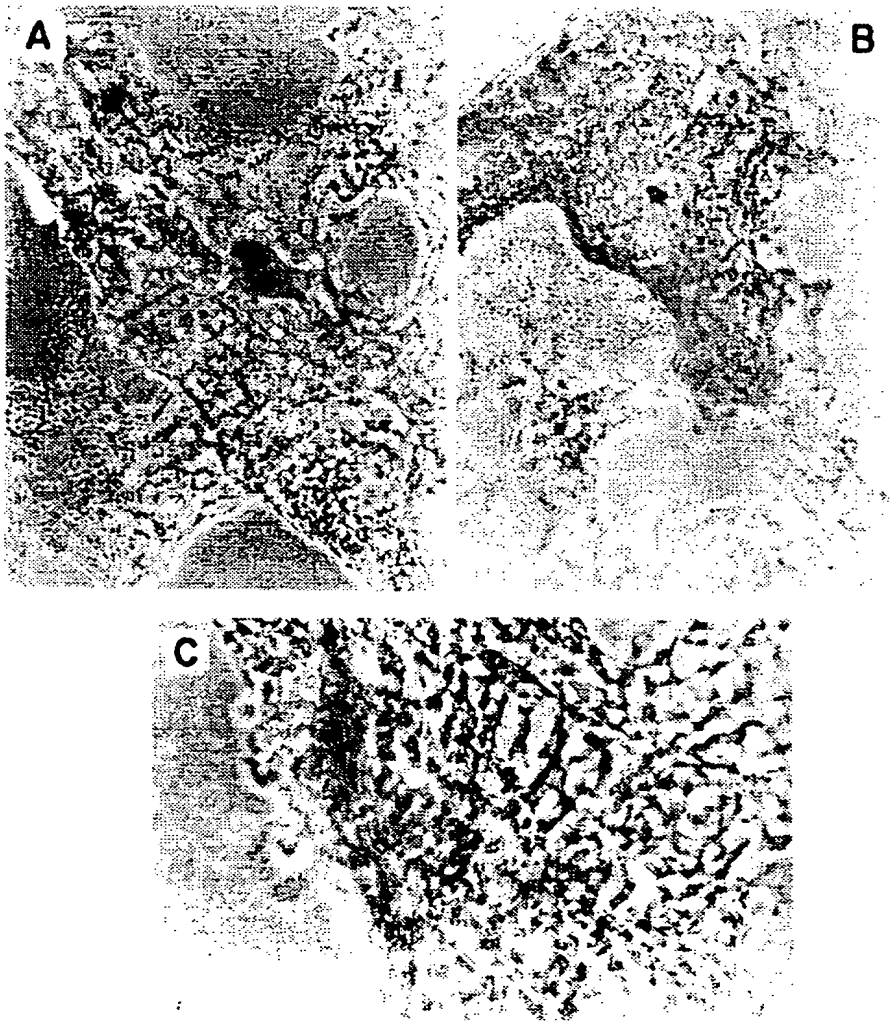


Fig_15

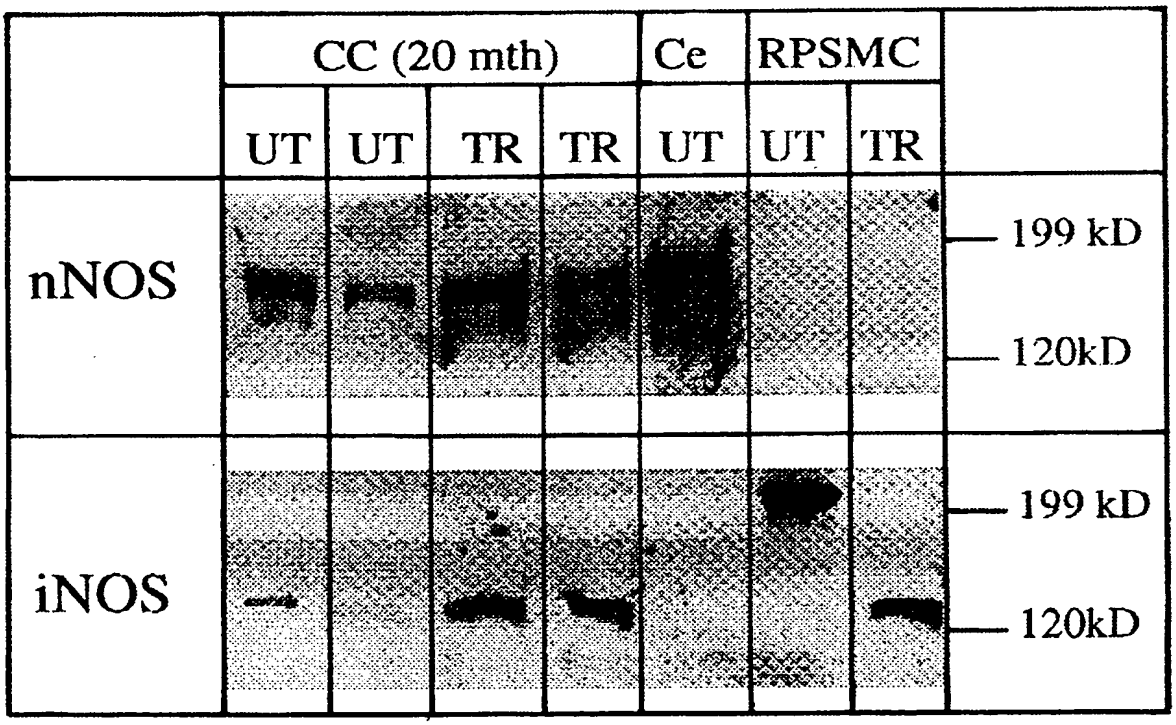

CC (20 mth)
Ce
RPSMC
UT
UT
TR
TR
UT
UT
TR
nNOS
199 kD
120kD
iNOS
199 kD
120kD

Fig. 16

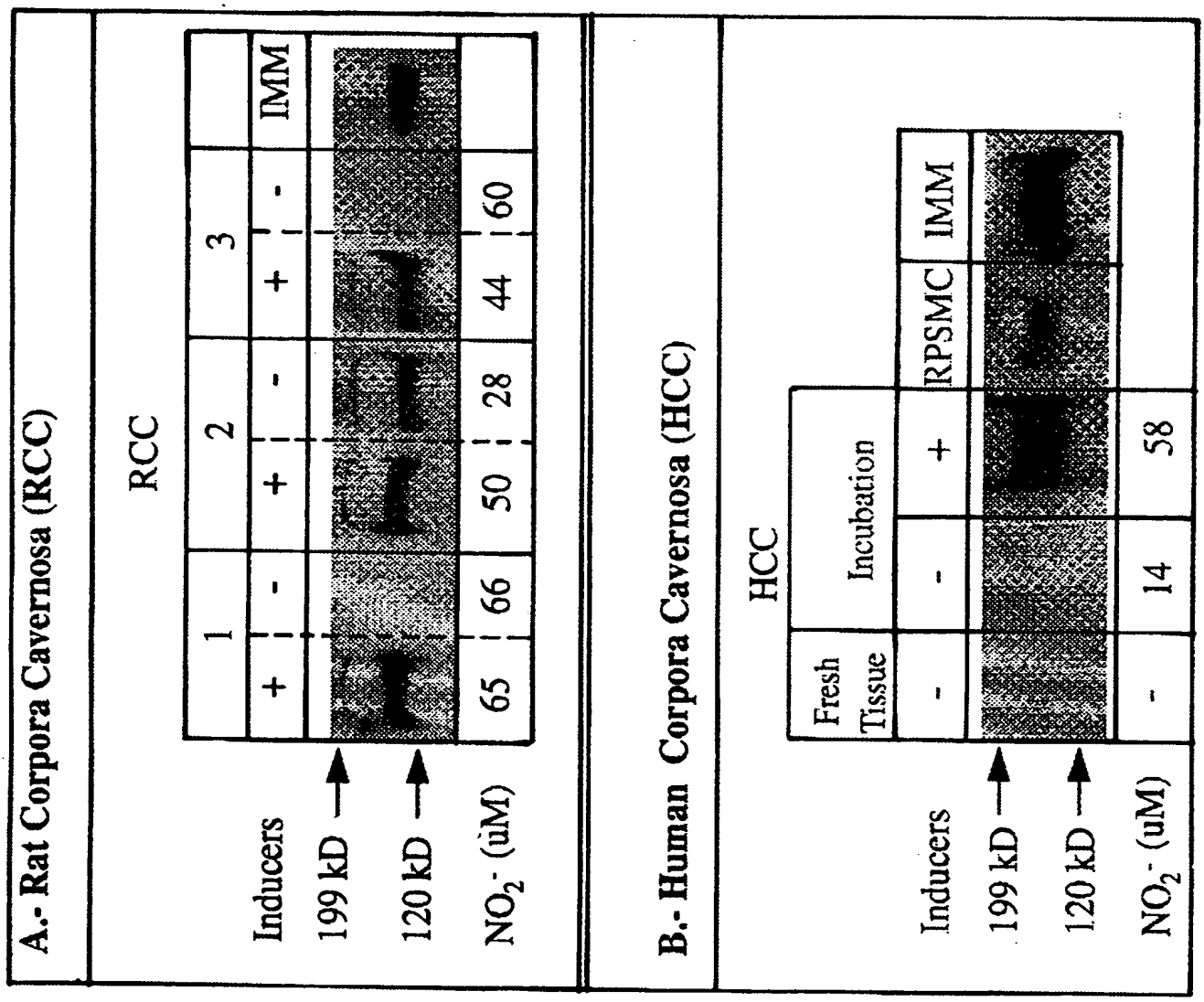

A.- Rat Corpora Cavernosa (RCC)
RCC
1
2
3
IMM
+
-
+
-
+
-
Inducers
199 kD
120 kD
NO_2^- (uM)
65
66
50
28
44
60
B.- Human Corpora Cavernosa (HCC)
HCC
Fresh Tissue
Incubation
RPSMC
IMM
Inducers
199 kD
120 kD
NO_2^- (uM)
-
-
+
-
14
58

Fig. 17

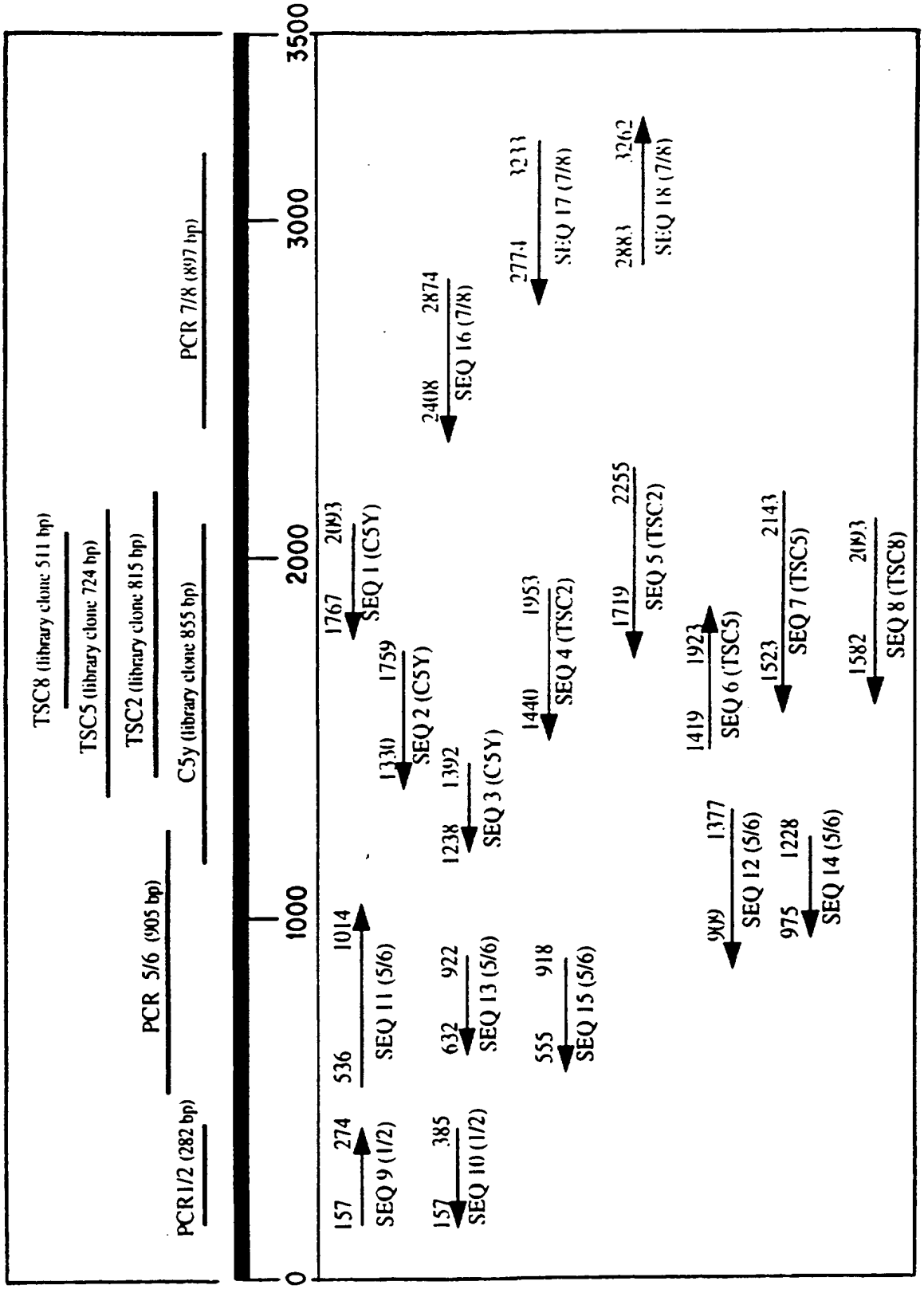

TSC8 (library clone 511 bp)
TSC5 (library clone 724 bp)
TSC2 (library clone 815 bp)
C5y (library clone 855 bp)
PCR 7/8 (897 bp)
PCR 5/6 (905 bp)
PCR1/2 (282 bp)
0
1000
2000
3000
3500
157 274 SEQ 9 (1/2)
157 385 SEQ 10 (1/2)
536 1014 SEQ 11 (5/6)
632 922 SEQ 13 (5/6)
555 918 SEQ 15 (5/6)
1767 2093 SEQ 1 (C5Y)
1330 1759 SEQ 2 (C5Y)
1238 1392 SEQ 3 (C5Y)
1440 1953 SEQ 4 (TSC2)
1719 2255 SEQ 5 (TSC2)
1419 1923 SEQ 6 (TSC5)
1523 2143 SEQ 7 (TSC5)
1582 2093 SEQ 8 (TSC8)
909 1377 SEQ 12 (5/6)
975 1228 SEQ 14 (5/6)
2408 2874 SEQ 16 (7/8)
2774 3233 SEQ 17 (7/8)
2883 3262 SEQ 18 (7/8)

Fig.18

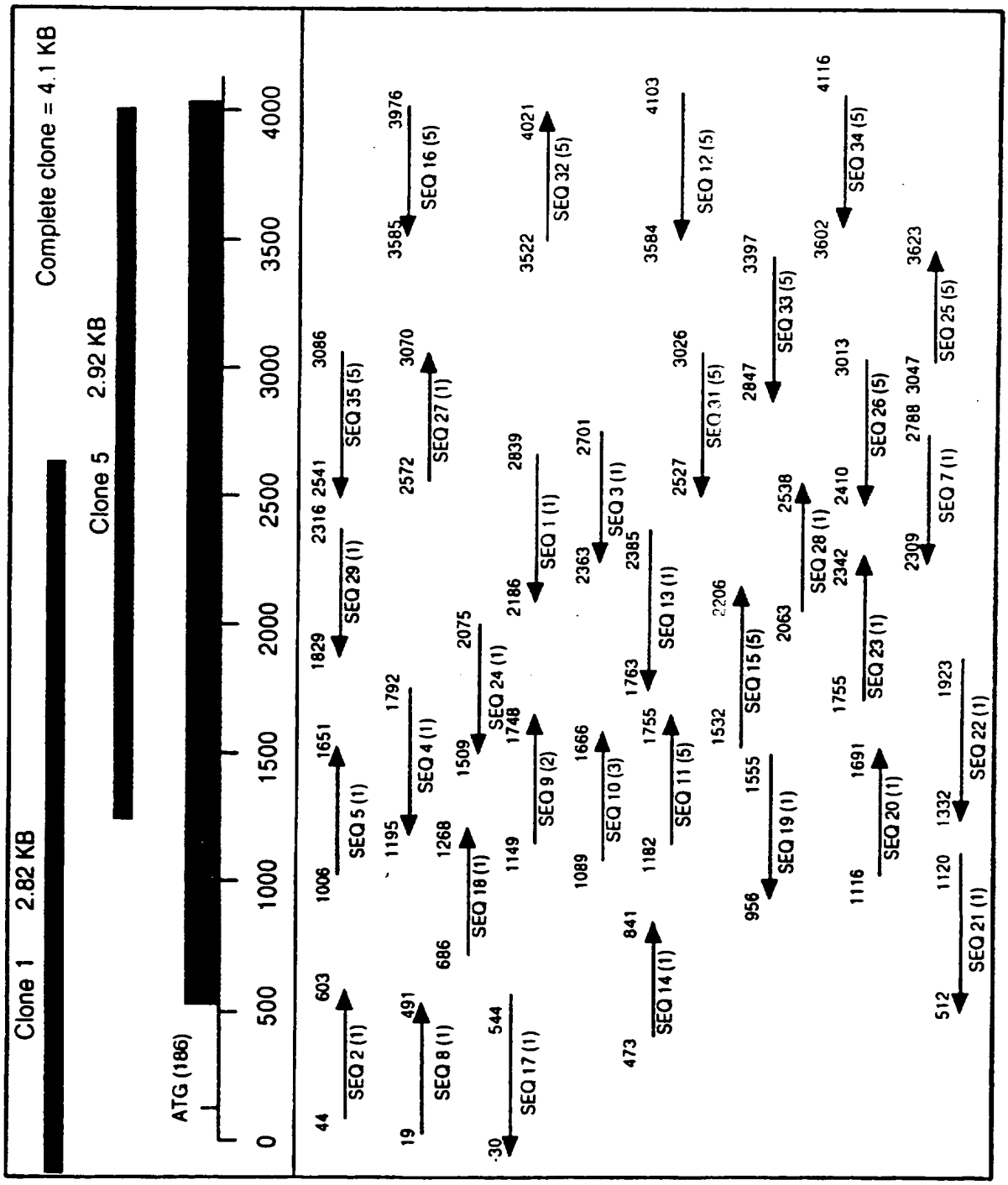

Clone 1 2.82 KB
Complete clone = 4.1 KB
Clone 5 2.92 KB
ATG (186)
0
500
1000
1500
2000
2500
3000
3500
4000
SEQ 2 (1)
SEQ 8 (1)
SEQ 17 (1)
SEQ 14 (1)
SEQ 18 (1)
SEQ 5 (1)
SEQ 4 (1)
SEQ 9 (2)
SEQ 10 (3)
SEQ 11 (5)
SEQ 24 (1)
SEQ 19 (1)
SEQ 20 (1)
SEQ 29 (1)
SEQ 1 (1)
SEQ 3 (1)
SEQ 13 (1)
SEQ 15 (5)
SEQ 23 (1)
SEQ 22 (1)
SEQ 21 (1)
SEQ 28 (1)
SEQ 35 (5)
SEQ 27 (1)
SEQ 31 (5)
SEQ 33 (5)
SEQ 26 (5)
SEQ 7 (1)
SEQ 16 (5)
SEQ 32 (5)
SEQ 12 (5)
SEQ 34 (5)
SEQ 25 (5)

Fig_19

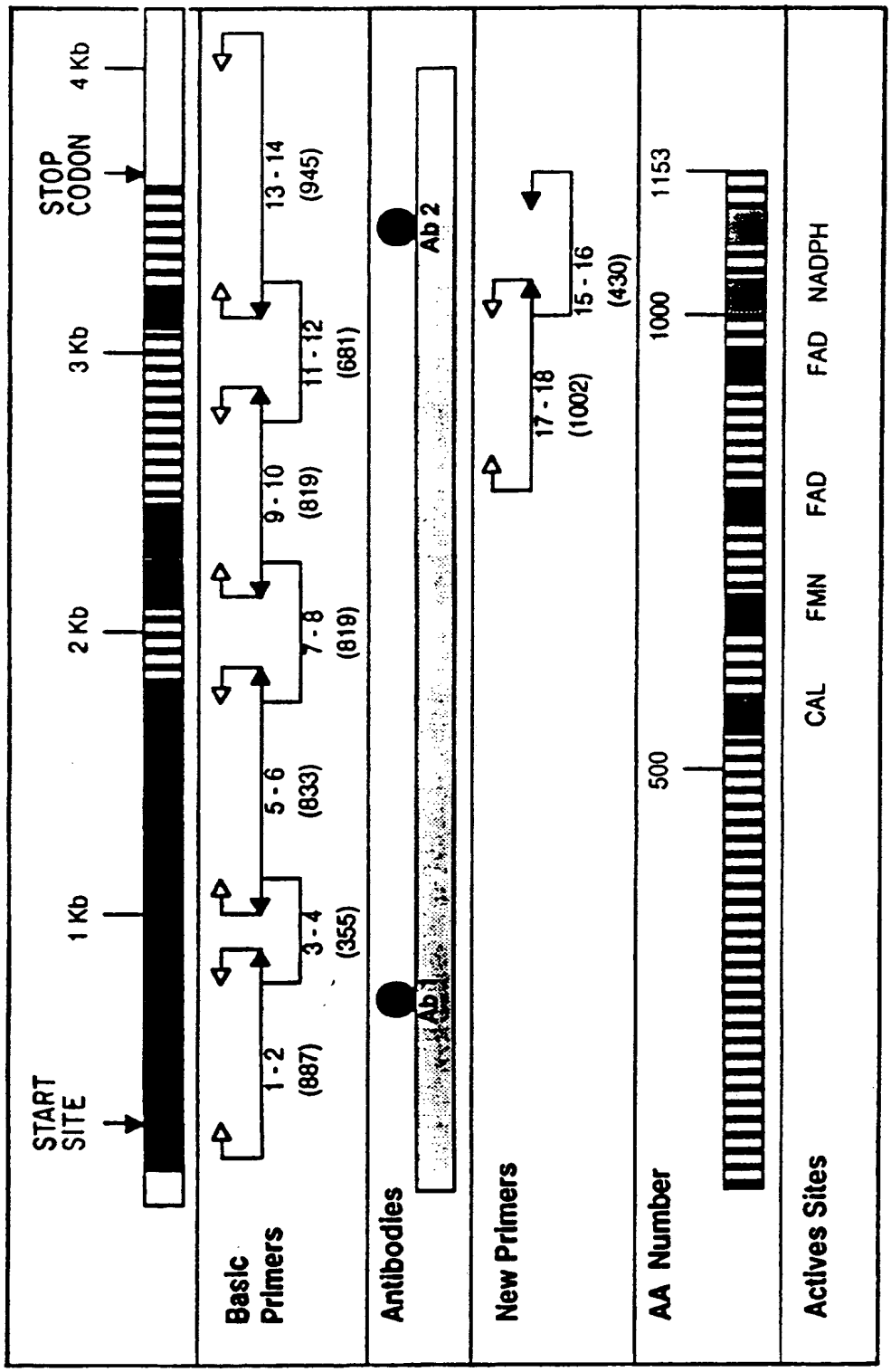

START SITE
1 Kb
2 Kb
3 Kb
STOP CODON
4 Kb
Basic Primers
1 - 2 (887)
3 - 4 (355)
5 - 6 (833)
7 - 8 (819)
9 - 10 (819)
11 - 12 (681)
13 - 14 (945)
Antibodies
Ab 1
Ab 2
New Primers
17 - 18 (1002)
15 - 16 (430)
AA Number
500
1000
1153
Actives Sites
CAL
FMN
FAD
FAD
NADPH
: RT/PCR Primers
: 0.1 Kb
Values In Parenthesis: Fragment Size (bp)
: Sequenced
: Pending

Fig_20

FRG.#	LENGTH (bp)		5'-->3'(bp)	PRIMERS		RESTR. ENZYMES	
	PCR	RE	PCR	5'	3'	5'	3'
1	887	767	11 - 898	H1	H2	Xba I	Bam HI
2	355	245	762 - 1117	H3	H4	Bam HI	Eco RI
3	833	751	1018 - 1851	H5	H6	Eco RI	Ava I
4	513	319	1733 - 2246	H7	H8	Ava I	Bcl I
5	819	622	1998 - 2817	H9	H10	Bcl I	Bgl I
6	681	573	2694 - 3375	H11	H12	Bgl I	Sph I
7	945	845	3267 - 4112	H13	H14	Sph I	Hinf I
8	1002		2306 - 3308	H17	H18		
9	430		3289 - 3719	H15	H16		

Fig_21

FRAGMENT	PRIMER	BASE SEQUENCED	FROM/TO	% HOMOLOGIES/HH
H1/H2	1	473	34/507	97.3
H1/H2	2	255	615/867	89.7
H3/H4	3	325	783/1108	87.4
H3/H4	4	328	768/1095	93.6
H5/H6	5	464	1041/1505	92.9
H5/H6	6	375	1446/1820	94.6
H9/H10	9	338	2022/2360	96.5
H9/H10	10	369	2004/2371	98.6
H12/H13	12	88	3265/3352	87.1

Fig_22

HH: Human Hepatocyte

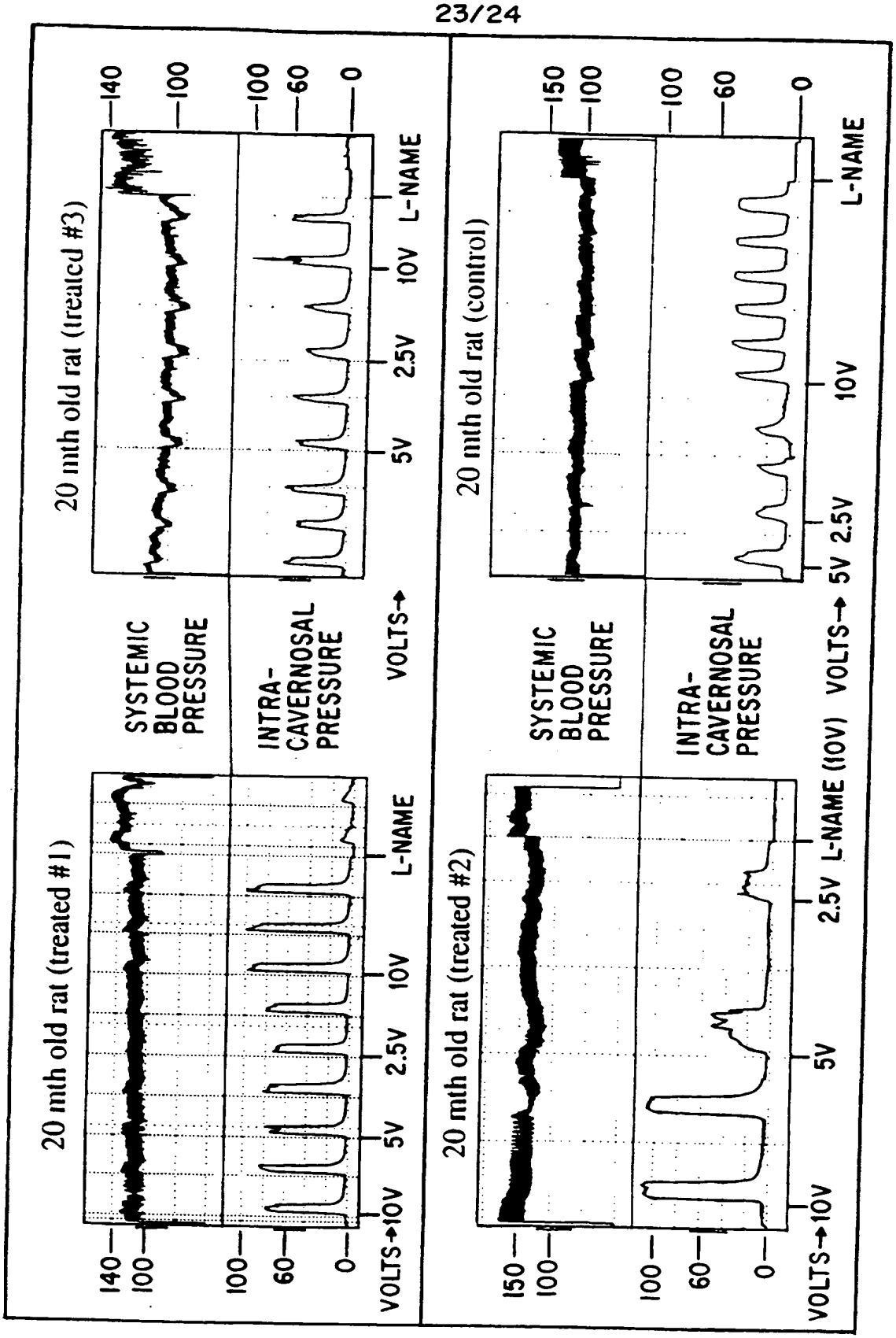
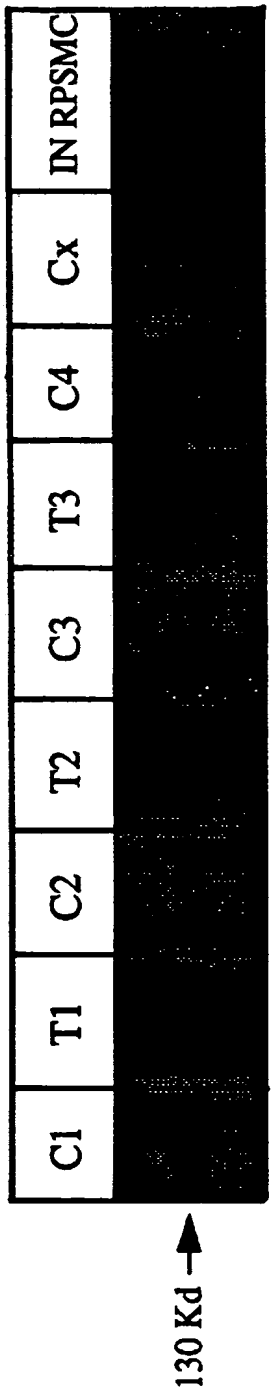

20 mth old rat (treated #1)
SYSTEMIC BLOOD PRESSURE
INTRA-CAVERNOSAL PRESSURE
VOLTS→
L-NAME
20 mth old rat (treated #3)
20 mth old rat (treated #2)
20 mth old rat (control)
VOLTS→10V 5V 2.5V 10V
2.5V L-NAME (10V)
5V 2.5V 10V

Fig.23



C: 20 mth control rats

T: 20 mth treated rats

Cx: 5 mth castrated rat, treated

IN RPSMC : induced rat penis smooth muscle cells

Fig. 24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14588

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 59/00
US CL :424/718

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/718

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN CAS File CA/MEDLINE/BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Chemical Abstracts, issued 1994, Ehren et al, "Nitric Oxide Synthase Activity in the Human Urogenital tract" Abstract No. 122:259288.	1-30

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 28 FEBRUARY 1996	Date of mailing of the international search report 20 MAR 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PAUL J. KILLOS tcj Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)*